University of Alberta

Antigen And Cytokine Modulation Of Autoimmune Diabetes In The NOD Mouse

by

Dean Kirkman Smith

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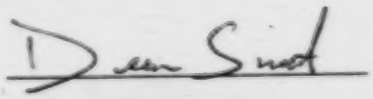

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Antigen and Cytokine Modulation of Autoimmune Diabetes in the NOD Mouse** submitted by **Dean Kirkman Smith** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Medical Sciences (Immunology)**.

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Date: May 8, 1998

Abstract

My first objective was the cloning, sequencing and immunological evaluation of the *Escherichia coli* protein glutamic acid decarboxylase (ecGAD). EcGAD had previously been shown to prevent diabetes in the non obese diabetic (NOD) mouse and was hypothesized to be cross-reactive with the mammalian GAD enzymes, which are central autoantigens in autoimmune diabetes. My second objective was to evaluate the recombinant adenovirus mediated local expression of type 2 cytokines that are associated with diabetes prevention (i.e. IL-4 and IL-10) in an islet transplant model in the NOD mouse.

Two GAD structural genes, designated *gadA* and *gadB* were identified and mapped to position 4046 kb and 1588 kb respectively on the *E. coli* chromosome. The sequences were found to be 98% similar at the nucleotide level and were observed in all *E. coli* ECOR strains examined. Each gene encoded 466 residue polypeptides named respectively GAD α and GAD β, which differed by only 5 amino acids. Both GAD α and GAD β contain amino acid residues which are highly conserved among pyridoxal dependent decarboxylases, but otherwise the protein sequences were found to have low similarity with other GAD proteins including the mammalian GADs.

In vivo studies with purified ecGAD confirmed the original observations regarding disease prevention in NOD mice. However, the same was true of a control protein and we concluded these effects were related to non-specific modulation of the NOD immune system.

Preliminary adenoviral work with a ß galactosidase reporter construct in Balb/c mice demonstrated: efficient gene transfer with 50% of cells transduced at 2.5 $x10^3$ PFU/islet, no significant effect on the islets in glucose challenge assay and detectable in vivo expression for 8 weeks. NOD islets transduced with adenoviral vectors expressed either IL-4 or IL-10 in large quantities in vitro. However, when transduced islets were

transplanted in vivo, this cytokine expression did not prevent disease recurrence. This later work demonstrated that adenoviral vectors are an effective expression system for islets but that IL-4 and IL-10 were unable to down regulate an autoimmune attack.

To Kirsten for all that she makes possible.

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List of Abbreviations

AA	amino acid
Ag	antigen
APC	antigen presenting cell
Arg	arginine
Asp	asparagine
BCG	bacillus of Calmette and Guerin
bp	base pairs
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CMV	cytomegalovirus
CPM	count per minute
Cy	cyclophosphamide
DME	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
EDTA	ethylene-diamine tetra acetic acid
FP	footpad
FPLC	fast protein liquid chromatography
GAD	glutamic acid decarboxylase
GM-CSF	granulocyte-monocyte colony stimulating factor
HIP	human insulin promoter
HLA	human leukocyte antigen
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IP	intraperitoneal
IV	intravenous
Kb	kilo base
kD	kilo Dalton
KO	knock out
LPS	lipopolysaccharide
LMP	low melting point
MBP	myelin basic protein
Mϕ	macrophage
MHC	major histocompatibility complex
MOI	multiplicity of infection
NK	natural killer (cells)
NOD	non obese diabetic
NOD/Alt.	non obese diabetic mice (University of Alberta)
NOD/lt	non obese diabetic mice from the Leiter colony (Jackson Labs)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PPD	purified protein derivative
PT	Pertussis toxin
RIP	rat insulin promoter
SDS	sodium dodecyl sulphate
Ser	serine

SMRI	Surgical Medical Research Institute
TCR	T cell receptor
TE	tris EDTA buffer
Th	T helper
TNF	tumor necrosis factor
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Chapter 1

General Introduction

The chronic breakdown of tolerance to self antigens results in either tissue specific or generalized autoimmunity, typically with an undefined etiology (Ridgway et al., 1994). Diabetes can divided into two main types, both of which manifest as elevated blood glucose in affected individuals. Type I diabetes is generally considered as the archetypal tissue specific autoimmune disease (Castano and Eisenbarth, 1990; Tisch and McDevitt, 1996; Delovitch and Singh, 1997). This more severe form of the disease is caused by the specific destruction of the insulin producing ß cells in the pancreas by immune effectors and their products. The result is a life-long dependency on supplemental insulin to prevent ketoacidosis and death in both diabetic humans and animal models. The requirement for exogenous insulin defines this form of the disorder as insulin dependent diabetes mellitus (IDDM) and the autoimmune mechanism of ß cell destruction gives it its type 1 designation (Keen and Barnes, 1997).

The paradox of type 1 diabetes: The riddle posed to anyone venturing into research in autoimmune diabetes is the following: Given that the principle susceptibility factors are particular MHC class II molecules, present on all professional antigen presenting cells (APCs) throughout the body, how does this state of affairs give rise to a tissue specific autoimmune response? The experimental work presented in this thesis examines the role of microbial antigens and host cytokines in the modulation of autoimmune diabetes in the NOD mouse. However, recent work by several groups provides a new basis for our understanding of the role of MHC in autoimmunity, as well as providing insight into novel contributing factors beyond MHC. Some of this work will be highlighted in this

introduction and integrated into the final discussion in an attempt to resolve the apparent paradox described above

Disease parameters in humans: IDDM is also known as juvenile diabetes which speaks to the typical age of onset in humans. Patients can present with type 1 diabetes at birth, and incidence tends to peak just prior to the age of 6 and again at puberty (Staines et al., 1993). However, in some studies up to 10 % of patients that were classified as recent onset type 1 diabetics were over the age of 65 (Kilvert et al., 1984; Bates, 1986). In North America and Europe IDDM patients generally represent 15 to 25 % of the diabetic population (Gatling et al., 1988), with the remainder being classified as non insulin dependent (NIDDM) or type 2 diabetics. Although some forms of NIDDM also have an autoimmune component (Rowky et al., 1992; Atkinson and Maclaren, 1994) and therapy can involve insulin administration, these patients are typically treated with modifications in diet and ketoacidosis is not an issue. The continued focus on IDDM in diabetes research in spite of the long standing availability of insulin therapy (Banting et al., 1922) is largely driven by the morbidity associated with this disorder. Vascular complications observed in IDDM patients include myocardial infarction, blindness, peripheral neuropathy as well as kidney failure (The Diabetes Control and Complications Trial Research Group, 1993). In addition, the young age and perceived vulnerability of most newly diagnosed IDDM patients, faced with a lifetime of insulin injections and an early death from complications, has provided strong motivation for fund-raising efforts in aid of this research.

Prevalence of the disease: The overall incidence of IDDM varies widely in different regions of the world and even within individual countries (Karvonen et al., 1993), suggesting that both genetic and environmental factors contribute to the disease (discussed below). In Europe the incidence ranges approximately ten-fold amongst children below the age of 14, with Macedonia at 3 per 100,000 (Kocova et al., 1993) and Finland at 35 per 100,000

(Tuomilehto et al., 1992). The south to north trend toward increasing incidence of IDDM in Europe is now well established (Green et al., 1992) but the pattern is not entirely consistent. Sardinia (Muntoni et al., 1990) has a rate just below that of Finland, whereas Iceland, which is at a higher latitude, has only a moderate incidence comparable to that of Southern Europe (Helgason et al., 1992). North American populations of European descent are generally intermediate in their incidence of type 1 diabetes (Rewers et al., 1988; Tajima and LaPorte, 1992; Karvonen et al., 1993) while non-whites in the United States have a reduced incidence, and all Asian populations studied to date (i.e. China, Japan and Korea) have a low incidence, at < 1 per 100, 000 (Tajima and LaPorte, 1992; Japan IDDM Epidemiology Group, 1993; Wong et al., 1993; Ko et al., 1994; Hua et al., 1994). In the few countries in Europe and Scandinavia that have kept long term records it is interesting to note that there has been a general increase in the disease incidence over the past few decades (Joner and Sovik, 1989; Soltesz et al., 1990; Tuomilehto et al., 1991; Dahlquist and Mustonen, 1994).

From endocrinology to autoimmunity: Although IDDM is now widely considered to be a multi-genic autoimmune disorder potentially influenced by a variety of environmental factors (Castano and Eisenbarth, 1990; Tisch and McDevitt, 1996; Delovitch and Singh, 1997), this represents a relatively recent view of the disease. The first hint of immune involvement actually came prior to the discovery of insulin (Banting et al., 1922) with the observation of an inflammatory process occurring around the pancreatic islets (later termed insulitis) in patients who had died of diabetic ketoacidosis (von Meyenburg, 1940). However, this thread was not picked up again until 1965 when the original observation was confirmed in 15 of 22 IDDM patients who had died within 6 months of diagnosis (Gepts, 1965). Beyond this and other histological evidence, the case in support of IDDM as an autoimmune disease comes from the following: i) the observation that diabetes often occurs together with other autoimmune conditions (Dreu and Notkins, 1987), ii) the

recognition, initially of a HLA class I association, and later of an even stronger class II association with IDDM (Singal and Blajchman, 1973; Nerup et al., 1974; Plaz et al., 1981; Nepom, 1993; Sanjeevi et al., 1995), iii) the discovery of anti-islet cell antibodies (ICA) in the serum of pre-diabetics and of newly diagnosed patients, (Bottazzo et al., 1974; Lernmark et al., 1980), and iv) the discovery of circulating antibodies against specific islet cell antigens (Baekkeskov et al., 1982; Palmer et al., 1983; Baekkeskov et al., 1990; Atkinson and Maclaren, 1993). Other convincing lines of evidence include: i) the observation that cyclosporin A could slow the rate of islet destruction and prolong the honeymoon (temporary remission) in newly diagnosed patients (Sutherland et al., 1984; Udeinya et al., 1983; the Canadian-European Randomized Control Trial Group, 1988), ii) the observation that rapid ß cell destruction occurred in a disease free pancreas which was transplanted into a HLA identical diabetic twin (Sibley et al., 1985), and iii) the observation that IDDM was induced when bone marrow was transplanted from an affected patient to an HLA identical non-diabetic twin (Lampeter et al., 1993). The understanding of autoimmunity in diabetes has also been greatly aided by animal models of the disease such as the non-obese diabetic (NOD) mouse and the bio breeding (BB) rat, both of which display many of the features of type 1 diabetes seen in humans (Rossini et al., 1995). These models are presented in more detail below.

Genetics versus environment in IDDM: In humans, at least 20 chromosomal regions have been linked to susceptibility for type 1 diabetes (Bain et al., 1997). However, most attention has focused on the MHC, and especially on the class II region. Class II MHC molecules consist of αβ heterodimers which present antigens to T cells. In humans there are three such groups of molecules available, denoted as DP, DQ and DR. Within Caucasian populations the DQ alleles confer either susceptibility or resistance to disease. For example, there is a five to thirty-five fold increased risk of IDDM in individuals who are heterozygous for DQ2 (DQA1*0501; DQB*0201) and DQ8 (DQA1*0301;

DQB1*0302). In contrast, a single dose of DQ6 (DQA1*0102; DQB1*0602) is associated with resistance to disease (Thorsby and Ronnigen, 1993). In this nomenclature the A and B refer to the α and ß chain of the DQ molecules respectively and the four digit number refers to a specific allele of one of the chains, each of which is now known at the nucleotide level.

The increased susceptibility that the DQB1*0302 tends to confer in Caucasians has been further linked to the lack of an aspartic acid (Asp) residue at position 57 of the DQß chain (Todd et al., 1987). Within this group 96% of diabetics but only 19% of healthy controls are non-Asp57 on both DQ alleles (Todd et al., 1987), and non-Asp 57 is also found in the class II molecules present in both the NOD mouse (Acha Orbea and McDevitt, 1987) and the BB rat (in both these strains residue 57 is Ser, whereas it is Asp in disease-free strains). However, it should be noted that in certain populations lack of Asp at position 57 may be a marker of disease susceptibility rather than a requirement, since 72% of Japanese type 1 diabetics are homozygous for Asp at position 57 of DQß (Khalil et al., 1990), and non-Asp substitutions at a combination of positions 56 and 57 in NOD mice protect against diabetes (Lund et al., 1990; Miyazaki et al., 1990; Slattery et al., 1990).

The mechanism by which MHC molecules in general might confer susceptibility or resistance to IDDM is still somewhat speculative. One mechanism which has been suggested depends on the role these molecules play in ensuring deletional tolerance through negative selection in the thymus (Sheehy, 1992). According to this model, MHC alleles that correlate with resistance are capable of binding diabetogenic peptides with high affinity, resulting in effective negative selection of potentially self-reactive T cell receptors (TCRs). The corollary is that MHC alleles that confer susceptibility have a low affinity for the same peptides, and this permits auto-reactive T cells to escape into the periphery. When the unique class II molecule (I-A^{g7}) of the NOD mouse was examined in light of this theory

it was found to be unstable in comparison to I-A^b and demonstrated weak and unstable peptide binding (Carrasco-Marin et al., 1996). Based on these results the authors speculated that deletional tolerance of potentially autoreactive T cells would be difficult to achieve with the I-A^{g7} class II.

Direct support for the role of certain class II molecules in the prevention of insulitis and diabetes has been obtained in NOD mice. Engineering the expression of the endogenous I-E heterodimer, which is normally not expressed in the NOD mouse due to a promoter defect in the I-Eα locus, prevented insulitis, but this was not found to be associated with deletional tolerance (Lund et al., 1990). Later work supported this observation and established that bone marrow-derived APCs were responsible for prevention of disease (Parish et al., 1993). However, deletional tolerance mediated by class II MHC has recently been demonstrated as a possible mechanism in prevention of type 1 diabetes in the NOD mouse, but the antigen or antigens involved remain a mystery (Schmidt et al., 1997).

As important as genetic factors such as MHC are for the development to type 1 diabetes, it is equally clear that this is only part of the story. This is illustrated by the relatively low concordance rates for IDDM amongst monozygotic twins (30-50%; (Creamer et al., 1983)). Therefore, genetic predisposition is a necessary but insufficient condition with respect to the development of type 1 diabetes, and the environment must also play a critical role.

Environmental factors in IDDM-pathogens: As reported by Gamble et al. (Gamble et al., 1969; Gamble and Taylor, 1969) viral infection and the seasonal nature of IDDM onset has been a topic of speculation and study since the early part of this century. Several viruses have under one or another circumstance been associated with the onset of clinical diabetes, and these include Coxsackie B virus (Gamble et al., 1969; Hyoty et al., 1995), rubella

virus (Mensen et al., 1978; Gamble, 1980), and cyomegalovirus (CMV) (Pak et al., 1988; Oldstone, 1988). As reviewed by Yoon (Yoon, 1990), several viruses have been shown to replicate in islets in vitro and cause destruction of ß cells in animal models, but generally without immune involvement. These and other viruses may potentially initiate or exacerbate autoimmunity through molecular mimicry of self antigens (Oldstone, 1988; Kostraba et al., 1992). Mimicry of self antigens is speculated to be a means by which microbes can evade immune surveillance, but under certain situations this can also lead to a breakdown of self tolerance and result in autoimmunity (Oldstone, 1987). For IDDM, "proof in principle" of the destructive potential of mimicry in autoimmunity was provided by transgenic mouse models.

In these experiments autoimmune ß cell destruction was driven by a virally derived "self" transgene expressed throughout life specifically on the pancreatic ß cells (Oldstone et al., 1991; Qari et al., 1992). However, autoimmunity was generally only triggered following infection by the same virus that was used as the source of the transgene. For human IDDM the question is, what endogenous islet antigens have been implicated as potential targets of molecular mimicry with cross-reactive microbial antigens?

Examples of potentially cross reactive epitopes and/or sequence similarities between viruses and islet antigens include the following: i) CMV and a 38 kD islet protein (Pak et al., 1990; Roep et al., 1991), ii) the ß cell expressed retroviral antigen p73 and insulin (Serreze et al., 1988), iii) rubella virus and a 52 kD islet antigen (Karounos and Wolinsky, 1993; Karounos et al., 1990) iv) Coxsackie virus B4 and a similar region of islet carboxypeptidase H (and interestingly, also the HLA-DQ 3.2 ß chain (Baum et al., 1995)), v) yet another region of Coxsackie virus B4 and glutamic acid decarboxylase (GAD 65) (Baekkeskov et al., 1990; Jones and Armstrong, 1995), and vi) mycobacterium heat shock protein and human Hsp65 (Atkinson and Maclaren, 1993). While all of these cross

reactivities have been demonstrated at the level of antibodies, and T cell cross reactivity may have been shown between GAD65 and Coxsackie B4 (Atkinson et al., 1994) no direct link has been established between any of these pathogens and IDDM initiation.

The first direct evidence of molecular mimicry leading to autoimmunity involving a true self antigen was recently provided in a mouse model of herpes stromal keratitis (HSK). In this disease infection with herpes simplex 1 (HSV-1) results in T cell mediated destruction of the corneal tissue and blindness in both the mouse model and in humans (Zhao et al., 1998). The HSV-1 genome encodes an amino acid sequence (UL6) that mimics a polypeptide also expressed in the cornea. Infection with wild-type HSV-1 induces HSK in up to 85% of animals, whereas infection with a UL6-deficient HSV-1 reduced the incidence of HSK to near zero (Zhao et al., 1998). A similar direct link between infection with a pathogen and disease induction has been lacking in IDDM. However, the above work and more recent studies involving a novel retroviral superantigen in humans will likely renew interest in this topic.

Superantigens are viral or bacterial products that stimulate T cells by binding MHC class II molecules and specific TCR Vß subunits, independent of the antigen specificity of the TCR (Choi et al., 1989; White et al., 1989). Conrad and co-workers (Conrad et al., 1997) have isolated a MMTV-like human retrovirus that encodes a superantigen expressed in new onset IDDM patients but not in healthy controls (ten of each were examined). The model proposed by this group has the superantigen, which was found to be expressed in lymphocytes but not in islet ß cells, inappropriately activating T cells bearing Vβ7, which then home to the islet where they initiate disease. However, both the authors (Conrad et al., 1997) and their critics (Benoist and Mathis, 1997) acknowledge that the data cannot discriminate between the superantigen as an initiator of disease, a downstream potentiating factor, or merely a marker of disease.

Endogenous ß cell tropic retroviruses have also been implicated in the induction of IDDM in humans (Yoon, 1997) and in the NOD mouse (Leiter and Wilson, 1988; Yoon, 1990; Hanafusa et al., 1996). In these studies retroviral particles were evident in the ß cells themselves, and at least in the mouse model production of viral particles was observed to peaked just prior to the initiation of insulitis (Hanafusa et al., 1996).

In contrast to the indirect and indirect evidence that infection by specific viruses can play a role in certain instances of IDDM pathogenesis, there is also a more counter-intuitive notion that exposure to certain non-specific pathogens (viral or bacterial) may actually prevent disease. For example, correlative evidence for this idea is provided by the previously mentioned south to north gradient of IDDM incidence in Europe (Green et al., 1992). Further, support for a potentially positive (i.e. "protective") role for early non-specific immune stimulation (perhaps to ensure balanced immune regulatory function) is provided by a study that examined hygiene in infancy and its impact on the later development of another autoimmune disorder, inflammatory bowel disease (Gent et al., 1994). This study suggested that Crohn's disease was five times more likely to occur in individuals that had had hot running water available during their infancy. This index is clearly a very indirect measure of the actual conditions of hygiene experienced by these individuals, but as will be seen below, there is also considerable evidence to support the idea that early immuno-stimulation in animal models of type 1 diabetes can block progression to disease. Finally, an additional confounding issue in the evaluation of environmental factors is the role of diet.

Dietary factors: Here again there is consensus on the potential importance of diet but no general agreement on the nature of the specific agents or the possible mechanisms involved (Yoon, 1997; Leiter, 1990). As an example of this, bovine serum albumin (BSA) found in

cow's milk, and specifically a linear epitope of this protein ("ABBOS"), has been suggested as a "triggering" antigen in IDDM (Karjalainen et al., 1992). The proposed immunological mechanism is based on a putative cross reactivity with the ß cell autoantigen ICA69 and ABBOS (Glerum et al., 1989; Martin et al., 1991), but these results have been controversial (Ronningen et al., 1998). While cow's milk may contain diabetogenic antigens or factors, when care is taken to prevent extraneous BSA contamination of islets preparations (from sources such as the bovine serum used in tissue culture), no antibody based cross reactivity between BSA and islet ICA69 is observed (Ronningen et al., 1998). However, recent work in the NOD mouse (Paxson et al., 1997) and the BB rat (Malkani et al., 1997), while supporting a role for dietary factors in protection from or susceptibility to disease, suggest that milk proteins, and BSA specifically, do not contribute to autoimmune diabetes. In fact, in one study dietary milk proteins were actually found to slightly decrease the incidence of disease (Paxson et al., 1997).

Islet autoantigens in IDDM progression: Not surprisingly the major antigens that have been associated with or linked to the progression of diabetes autoimmunity are those that are also candidates for molecular mimics. The rationale for the identification of islet autoantigens is based on the premise that as a tissue specific T cell mediated disease (Castano and Eisenbarth, 1990; Tisch and McDevitt, 1996; Delovitch and Singh, 1997) there would likely be tissue specific antigens involved in the initiation and progression of the autoimmune process. These ß cell antigens may for example cross-react with a pathogen, but the etiology is not limited to this mechanism alone. Antibodies to certain of these antigens, such as insulin, proinsulin, or GAD65 typically precede IDDM onset by several years; but since these antibodies are also seen in some individuals that never develop type 1 diabetes, their predictive value for IDDM is limited (Atkinson and Maclaren, 1993; Bosi et al., 1993). It should also be noted that none of the antigens induces an antibody response in all patients, and that only by simultaneously screening for antibodies to several of these

antigens can their predictive value be enhanced (Bingley et al., 1997). Of the antigen candidates identified, the only one that is known to be unique to ß cells is insulin or its precursors proinsulin and preproinsulin.

Human preproinsulin is synthesized as a single chain polypeptide with the signal peptide being cleaved and proinsulin being co-translationally delivered into the ER of the ß cell. Two cystine bridges fix the single chain into an overlapping head to tail circular configuration forming mature proinsulin (Howell, 1997). The central portion of the proinsulin molecule is then cleaved away resulting in the cystine linked 21 and 30 amino acid A and B chains of bioactive insulin along with the liberated C peptide.

Prior to insulin administration, 50% of newly diagnosed type 1 diabetics are observed to have insulin autoantibodies (IAA). In humans, IAA epitopes are found in both the A and B chains (Castano et al., 1993), while in the NOD mouse the dominant T cell epitope exists between residues 9-23 of the B chain (Daniel et al., 1995) and diabetogenic T cells cloned from insulitis lesions are responsive to this same peptide (Wegmann et al., 1994). Prior to disease, oral tolerization to insulin (Zhang et al., 1991) or immunization with inactive B chain (Muir et al., 1995) or peptide (Daniel and Wegmann, 1996) will reduce insulitis and diabetes in NOD mice. Based on these results, oral tolerance trials using insulin in prediabetic humans have been initiated (Dupre et al., 1997).

A recent result that highlights the potential benefits of islet antigen specific tolerance strategies demonstrated that expression of proinsulin under the control of a MHC class II promoter in transgenic NOD mice reduced insulitis to near background levels and completely prevented diabetes (French et al., 1997). These proinsulin II transgenic NOD mice were highly resistant to cyclophosphamide induced diabetes, whereas wild-type NOD mice respond to this drug with rapid onset of diabetes (see below). The concept behind

this transgenic experiment was that the class II promoter would ensure that sufficient proinsulin was expressed in the thymus to promote effective negative selection of any potentially autoreactive T cells. However, primed T cell responses to either insulin or an OVA control peptide could easily be detected in peripheral lymphocytes from these mice. Given the near absence of insulitis in these animals, they could be considered essentially free from autoimmunity, yet this was apparently not due to deletional tolerance. For some reason the insulin reactive T cells did not make a response against their (readily accessible) target tissue. This situation is not unique in transgenic models, and it is reminiscent for example of results obtained by C. Goodnow using HEL/IgHEL double-transgenic mice (Miller and Heath, 1993; Sprent et al., 1995).

An intriguing result with respect to insulin as an autoantigen in NOD mice comes from the work of Lipes and co-workers (Lipes et al., 1997; Kagi et al., 1997). In these studies, proinsulin was expressed as a transgene in a subset of pituitary cells in NOD mice. At this site the insulin-expressing pituitary cells were not a target of autoimmune attack even though ß cell destruction was occurring in the pancreas of the transgenic animals. This remained the case even if the insulin expressing pituitary cells were transplanted into fully diabetic non-transgenic syngeneic recipients, which resulted in "near normalization" of blood glucose (Lipes et al., 1997; Kagi et al., 1997). Here the context of the autoantigen or possibly other cellular co-factors seem to dictate the nature of the response to it.

Another diabetes autoantigen which has been the focus of intensive research was in fact the first specific antigen to be described beyond insulin. It was initially referred to as the 64 kD autoantigen, but was later identified as GAD (Lernmark and Baekkeskov, 1981; Baekkeskov et al., 1990). There are two isoforms of GAD, denoted as GAD65 and GAD67, and these are encoded by distinct genes (Bu et al., 1992). As reviewed in Faulkner-Jones et al. (Faulkner-Jones et al., 1996), in humans GAD67 is the principle

enzyme responsible for synthesizing the inhibitory neurotransmitter GABA in the central nervous system (CNS). GAD65 is the principal form expressed in human islets, although it also occurs widely in the CNS, particularly in the cerebellum, in addition to other non-neural tissues (Faulkner-Jones et al., 1996). In the mouse, GAD67 is the dominant islet isoform (Faulkner-Jones et al., 1996). The function of GAD in islets is still unresolved but clearly its expression is not unique to β cells or even to islet cells in general (Faulkner-Jones et al., 1996). With respect to function, it is interesting to note that GAD occurs primarily in neural tissues, but that it also occurs in islets, which are of neuroendocrine origin.

If GAD is central to the etiology of type 1 diabetes, as has been suggested, it again begs the question as to how autoimmunity is seen specifically in the islets and generally not in other GAD-containing tissues. However, in humans there is an extremely rare autoimmune condition known as Stiff Man Syndrome, in which GAD in the central nervous system does appear to be the focus of immune attack. Interestingly this disorder frequently occurs together with type 1 diabetes and it was this concordance that first suggested to investigators that GAD and the 64 kD autoantigen might be related (Baekkeskov et al., 1990).

Fifty to 90% of new onset type 1 diabetics have anti-GAD65 auto-antibodies, depending on their sex and age at diagnosis (Falorni et al., 1995; Vandewalle et al., 1995). Beyond its potential predictive value for IDDM, part of the initial interest in GAD was due to the identification of a potentially cross reactive epitope with Coxsackie virus B4 (Kaufman et al., 1992). This was followed by work that suggested that GAD65 T cell responses were present in young unprimed NOD mice, and that GAD immunization would greatly reduce insulitis and prevent diabetes when administered early in life as a soluble antigen in the absence of adjuvant (Kaufman et al., 1993; Tisch et al., 1993). Prevention

of diabetes with GAD has also been shown by early immunization with the protein (Elliott et al., 1994) or peptide (Tian et al., 1996a), by immunization with the protein at the time of IDDM onset (Tian et al., 1996b) and by consumption (i.e. oral tolerance) of GAD expressed in transgenic plants (Ma et al., 1997).

Indirect support for the notion of viral antigenic mimicry with GAD has been provided by the observation that 25% of newly diagnosed IDDM patients (but 0% of controls) were found to have T cell responses to a GAD peptide that is highly similar to a region of the P2-C protein of Coxsackie B virus. These same individuals also responded to the corresponding viral peptide (Atkinson et al., 1994). However, since these proliferation assays were performed on bulk cell populations, the results could indicate either dual responses in the population or cross reactivity. Another "twist" on the potential importance of both GAD and proinsulin as candidate autoantigens comes from the observation that the two proteins themselves share a region of similarity, and are potentially cross reactive (Rudy et al., 1995a; Rudy et al., 1995b; Brusic et al., 1997).

Interest in GAD (particularly GAD65) as a key autoantigen continues, although it is important to note that unlike insulin, diabetogenic T cell clones specific to GAD have not been isolated from NOD mice (Haskins and Wegmann, 1996). Therefore, GAD is unlikely to be involved in the earliest phase of the inflammatory process leading to autoimmunity, at least in the NOD mouse (Eisenbarth, 1994; Faulkner-Jones et al., 1996). Another issue to be considered with GAD is the inconsistent results that have been obtained with GAD peptide tolerization in NOD mice (Cetkovic-Cvrlje et al., 1997). In this latter study, specific GAD peptides that had previously been shown to prevent diabetes in NOD mice when given intraperitoneally, were shown to accelerate disease when injected into the thymus. These results underscore the caution that must be exercised when considering clinical trials for Ag specific immune modulation.

Other diabetes-related autoantigens that have been identified include carboxypeptidase H, ICA69, IA-2α (ICA512), IA-2ß (phogrin), and the 38 kD autoantigen (reviewed in; (Atkinson and Maclaren, 1993; Bosi et al., 1993; Roep, 1996). To further complicate matters the antigens GAD, IA-2α, and IA-2ß have recently been shown to jointly constitute the "64 kD autoantigen", a designation which had been previously assigned to GAD alone (Noorchashm et al., 1997a). Despite this list of islet non-specific autoantigens, if one subscribes to the belief that type 1 diabetes is caused by the expression of a key islet antigen, then the most interesting antigens probably remain to be identified. In this context, it is clear from studies in NOD mice that although initial strong spontaneous T cell responses are seen to whole islets, these cannot be accounted for by the collective responses to all of the known islet autoantigens (Gelber et al., 1994). Some of these unknown islet antigens may in fact be primary "triggering antigens" or at least early targets. However, it is important to keep in mind that especially in humans, there is probably no single trigger of disease, (Eisenbarth, 1994) and that initiating factors may vary depending on the subtleties of genetics (in and outside of the MHC) as well as on environmental factors.

Animal models of IDDM: As indicated previously much of what has been learned about autoimmunity has come from animal models. In addition to the NOD mouse and the BB rat there are several more exotic animal models of spontaneous type 1 diabetes which include: i) the Celebes black ape (Macaca nigra) (Howard, 1972), ii) the non obese Keeshond dog (Kramer et al., 1980), iii) Chinese hamsters (Grodsky and Frankel, 1981), iv) a specific strain of guinea pig (Munger and Lang, 1973), and v) a specific strain of New Zealand white rabbits (Conaway et al., 1980). However, the majority of work is done in the NOD mouse (and congenic or transgenic variants), and to a lesser extent the BB rat. A brief

description of the BB rat model is be provided below before moving on to the NOD mouse, which is the model used in this thesis.

The BB rat: In the disease prone (DP) BB rat, type 1 diabetes occurs spontaneously in approximately 60% of the animals (Nakhooda et al., 1978). Disease typically occurs between day 60 and 120 of life, and is associated with a massive lymphocytic infiltrate in the islet (Logothetopoulos et al., 1984). As in humans, diabetes occurs with approximately the same frequency in both sexes (Logothetopoulos et al., 1984), and IDDM susceptibility is strongly linked to the specific class II gene (Like et al., 1982; Poussier et al., 1982; Guttman et al., 1983; Jackson et al., 1984; Buse et al., 1985; Herold et al., 1989), which is designated $RT1^{u}$. The BB rat is noted for severe lymphopenia, which is marked by a virtual absence of $CD8^{+}$ T cells (MacKay et al., 1986; Like et al., 1986) and a 90% reduction in circulating $CD4^{+}$ OX19 expressing cells (Jackson et al., 1984; Herold et al., 1989), abnormalities which are not seen in diabetic humans or in NOD mice. As is discussed in more detail below in relation to the NOD mouse, IDDM onset in the BB rat is delayed or prevented by a variety of non-specific immune stimulants such as lymphocytic choriomeningitis virus (LCMV) or complete Freund's adjuvant (CFA), and the highest disease incidence occurs in colonies with low pathogen exposure (Rabinovitch and Singh, 1994).

The NOD mouse: The non-obese diabetic (NOD) mouse was initially developed in Japan; this particular strain came about as a fortuitous accident of a breeding program whose original goal was to develop a mouse model of early cataract formation. A portion of these animals develop autoimmune diabetes spontaneously, which is characterized by glucosuria, polyuria, wasting, and ketoacidosis. One striking difference between diabetes in humans and diabetes in NOD mice is that disease occurs in the NOD at a much higher frequency (≈40% greater) in females than in males (Pozzilli et al., 1993). This has been shown to be

largely controlled by sex hormones. Early gonadectomy in males increases the incidence of IDDM, which is reduced again if the castrated males are maintained on testosterone (Hawkins et al., 1993). However, the interaction between sex hormones and other diseases susceptibility factors is as complex as other aspects of NOD mouse immunology. For example, diabetes occurred in 100% of females that were gonadectomized and maintained on testosterone, whereas maintenance of intact females on testosterone reduced the incidence of disease.

The incidence of diabetes in female NOD mice in various colonies around the world is highly variable, ranging from 5% to 90% by week 20 and 20% to 100% by week 30 (Pozzilli et al., 1993). This divergence reflects genetic drift, differences in diet and differential exposure to microbial challenge in each of the different colonies (Pozzilli et al., 1993). An important piece of the NOD puzzle is the correlation between the incidence of diabetes in these animals and exposure to pathogens or microbes in general. In several colonies with a high baseline incidence, when the animals are delivered by cesarean section and maintained under sterile conditions (i.e. gnotobiotic mice) the incidence rises to 100%. This implies that in the absence of any external immune stimuli due to microbes, all the necessary conditions exist within the NOD mouse to induce autoimmune destruction of the ß cells.

In terms of immuno-genetics, the NOD mouse has a single unique class II molecule, (reviewed by (Kikutani and Makino, 1992)) and the extended haplotype is designated as H-2 K^d, I-A^{g7}, I-E *null*, D^b. Groups involved with the genetic analysis of IDDM in the NOD mouse have assembled an ever increasing number of genetic regions linked to disease susceptibility (reviewed in (Ikegami et al., 1996; Ritzel et al., 1997)). Each locus is denoted as *Idd* followed by a unique number (e.g. *Idd*1 denotes the MHC class II region), and currently in excess of 13 loci are under investigation. The contributions of diet to

IDDM etiology in the NOD will not be expanded upon any further, and the role of microbial challenge in disease initiation and prevention is discussed below.

Although the NOD is primarily a model of type 1 diabetes, as in the human, additional autoimmune conditions are also observed. Tissues that routinely develop a lymphocytic infiltrate include the submandibular and lacrimal glands in females and males respectively, typically after 9 weeks of age (Kikutani and Makino, 1992). The thyroid gland is another site of autoimmunity, although this is less common (Wicker et al., 1992), and other tissues are occasionally observed to be infiltrated, including the adrenal glands, bowels, and testes (Noorchashm et al., 1997a). It is interesting to note that most of the tissues targeted for autoimmune attack are endocrine in nature.

Chemical induction of diabetes: Several different drugs can be used in animal models to either accelerate the onset of autoimmune diabetes, to induce a "type 1 like diabetes syndrome", or to simply eliminate the ß cells of the islet. Cyclophosphamide accelerates the autoimmune process in NOD mice by an unknown mechanism, but it is not directly toxic to the ß cells (Makino et al., 1980). At least part of its activity seems to be through the up-regulation of interferon gamma inducing factor (IGIF or IL-18), which is observed in NOD but not in Balb/c spleen derived macrophages (Mϕ). Streptozotocin and alloxan are both typically used as ß cell toxins to induce IDDM in mouse strains other than the NOD. The mechanism of action of these two drugs is also not well defined, but involves both metabolic and free radical damage to the ß cell and other cell types at higher doses (Bone and Gwilliam, 1997). Multiple low doses of streptozotocin induces insulitis and a syndrome like human IDDM, but it is not generally considered to be an appropriate model for spontaneous autoimmune disease (Bone and Gwilliam, 1997).

Cellular mechanisms of autoimmunity in the NOD mouse: The infiltrate that ultimately results in the specific destruction of NOD pancreatic ß cells (Fujita et al., 1982) begins with the recruitment of cells with Mϕ and dendritic cell markers at about three weeks of age (Jansen et al., 1994). By week 5, CD8 T cells are detected in the infiltrate, and this is followed by CD4 T cells, B cells, and NK cells (Jarpe et al., 1990; Jansen et al., 1994). The potential regulatory or effector function and the relative importance of each of these cell types is controversial. However, some of minimum requirements are being identified through the study of gene knock out (KO) strains of NOD mice.

The role of T cells in mediating islet destruction is well supported by adoptive transfer (Wicker et al., 1986; Christianson et al., 1993) and T cell depletion studies (Harada and Makino, 1986; Koike et al., 1987), where both CD4 Th cells and CD8 CTLs have been shown to be required (Wang et al., 1987; Like et al., 1986; Sibley and Sutherland, 1987; Miller et al., 1988). Using insulin receptor density (IR^{high}) as a marker, an additional subset of T cells has been identified to be highly diabetogenic (McInerney et al., 1996). Since IR density and not the activation state of the T cells identified this diabetogenic cell population, this may provide an alternate model to explain how specific T cells are targeted to the islet.

Initiation of insulitis and regulation of the subsequent autoimmune response is considerably more complex, but some basic concepts are clear. Antibodies are not likely to play a critical role except as a marker of an ongoing autoimmune response, since transfer of T cells but not antibodies results in insulitis and diabetes (Miller et al., 1988; Bendelac et al., 1987). An antigen presenting function is clearly essential in the autoimmune process, and that function is initially provided by either dendritic cells or Mϕ's (Jarpe et al., 1990; Jansen et al., 1994). In addition to the histological and flow cytometric evidence in support of this premise, chronic treatment with silica (which is toxic for phagocytes) prevents

insulitis in both the NOD mouse and the BB rat (Hanenberg et al., 1989; Oschilewski et al., 1985; Lee et al., 1988; Charlton et al., 1988), and blockade of Mϕ adhesion molecules prevents the escalation from insulitis to ß cell destruction (Hutchings et al., 1990). The sequence and regulation of subsequent events in the inflammatory process is more speculative. Given that $CD8^+$ infiltrating T cells precede $CD4^+$ cells (Jarpe et al., 1990), and that absence of class I essentially abolishes insulitis in NOD mice(Katz et al., 1993; Wicker et al., 1994; Serreze et al., 1994), it would seem that $CD8^+$ T cells are critical regulators and/or effectors of events that lead to islet destruction and IDDM. The recognition of T cytotoxic (Tc) subsets (Mosmann and Sad, 1996) and the observation that CD4 cells can be influenced by CD8 cells to suppress IL-4 production and control perforin mediated killing functions (Williams and Engelhard, 1997) further supports the role of CD8 cells as potentially important regulators as well as CTL effectors. However, this is probably not the whole story, since NOD $CD4^+$ spleen cells and some (but not all, e.g. (Peterson and Haskins, 1996)) $CD4^+$ NOD T cell clones are capable of inducing diabetes in the absence of CD8 T cells (Christianson et al., 1993; Reich et al., 1989; Haskins and McDuffie, 1990; Nakano et al., 1991; Bradley et al., 1992; Daniel et al., 1995; Katz et al., 1995; Peterson and Haskins, 1996).

Recently, activated $CD8^+$ T cell clones were also observed to rapidly induce diabetes when introduced into irradiated NOD-SCID recipients (Wong et al., 1996). On the other hand, since activation states and homing are so integrally related (Butcher and Picker, 1996), it can be argued that introduction of fully differentiated and activated clones or cells into a recipient displaying appropriate "targets" is not a true test of the diabetogenic potential of T cells (Verdaguer et al., 1997). Thus, in another series of experiments, an islet specific CD4, I-A^{g7} restricted TCR transgenic line was compared to a CD8, K^d restricted TCR transgenic line, each placed on the RAG-$2^{-/-}$ NOD background to remove endogenous TCR (Verdaguer et al., 1997). In this comparison it was found that while

both CD4 and CD8 TCR transgenic populations could induce IDDM, disease onset was considerably delayed and the incidence reduced in the CD8 TCR mice. While neither TCR specificity was defined, each TCR transgene had shown equivalent diabetogenic potential in wild type NOD mice (i.e. in the presence of normal CD4 and CD8 T cells as well as B cells (Verdaguer et al., 1997; Schmidt et al., 1997)). Since the original diabetogenic T cell clones (from which the TCR transgenes were taken) were considered representative of CD4 and CD8 T cells in insulitis lesions, the authors concluded that due to the diminished maturation, activation and homing capabilities of the TCR transgenic CD8 cells relative to the CD4, the critical initiator of inflammation in the islet was more likely to be a CD4 cell. However, an alternate interpretation could be that these results reflect the additional levels of regulation that CD8 cells face relative to CD4 cells (even in a $RAG^{-/-}$ environment), and the true measure of CD8 cells diabetogenic potential was as reported for the wild type NOD TCR transgenic experiments, where T cells are operating in a more normal context (Schmidt et al., 1997).

Looking beyond T cells, what role might B cells have in type 1 diabetes? In general, IgM deficient (Akashi et al., 1997; Serreze et al., 1996) or IgM depleted NOD mice showed (Noorchashm et al., 1997b) greatly reduced or absent insulitis, and this was reflected in a reduced incidence or absence of diabetes. However, in another IgM KO NOD line, 2 of 7 B cell negative mice developed insulitis and diabetes (Yang et al., 1997). The discrepancy between the previous three studies and the latter one is not readily apparent, but overall it seems clear that B cells do play a critical role in autoimmunity in the NOD mouse, presumable by providing very efficient (if not inappropriate) Ag presentation (Constant et al., 1995). A critical role for B cells in type 1 diabetes is further supported by the results with CD40L blockade in NOD mice, which abrogated insulitis and diabetes providing it was started before 4 weeks of age (Balasa et al., 1997). Anti-CD40L treatment

apparently did not induce new dominant regulatory cells or immune deviation, since there was no protection in co-transfer studies with diabetic spleen cells.

Once destructive insulitis is established, what are the potential effector mechanisms responsible for ß cell destruction? The results with class I deficient mice argue in favor of a direct role for CD8 effectors, but as has already been discussed, CD4 cells are also essential for efficient disease induction (e.g.(Christianson et al., 1993)). Two more NOD KO strains provide some answers. Perforin deficient NOD mice had equivalent insulitis to their wild type litter mates but a reduced incidence of diabetes at 1 year (16 % versus 77 %) (Kagi et al., 1997). However, more striking was that in NOD Fas deficient (lpr/lpr) mice no insulitis or diabetes was observed, and these animals were resistant to spleen cell challenge from diabetic NOD mice (Itoh et al., 1997). Given that a cascade of events is likely to be responsible for ß cell destruction, what else is known about immunological defects in the NOD mouse that might provide insights into the break down of self-tolerance?

Immunological defects in the NOD mouse: The consistent development of type 1 diabetes, sialitis and the other reported autoimmunities in the NOD mouse clearly demonstrate that this animal has a fragile, tenuous and poorly regulated state of tolerance to self antigens. In addition to the poor tolerization potential of the I-A^{g7} molecule that was referred to earlier, maintenance of peripheral tolerance is also defective in the NOD relative to other non-autoimmune strains, as demonstrated by immunization experiments with self antigens (Ridgway et al., 1996).

In considering factors that could be responsible for the lack of self-tolerance, one obvious place to start is with the APC. In fact NOD APCs are defective by a variety of criteria when compared to other strains. These defects include low "suppressor cell"

activity in a syngeneic mixed lymphocyte reaction (SMLR), low TNFα (Satoh et al., 1989; Jacob et al., 1990) and IL-1 expression, inappropriate class 1 and IFNγ responsiveness, signaling defects involving protein kinase C (Serreze et al., 1989; Leiter and Serreze, 1992; Serreze et al., 1993a; Serreze et al., 1993b), and defective expression of Fc gamma RII on Mϕ (Luan et al., 1996).

The NOD mouse is also noted for low expression of IL-2 and IL-4, the latter of which has been linked to poor homing of T cells (Cameron et al., 1997) and pathogenesis in NOD mice, since disease could be prevented by administration of IL-4 (Rapoport et al., 1993). The low IL-4 expression has been attributed to a reduced number of NK1+T-like cells in the NOD mouse and to impaired IL-4 production from these cells (Gombert et al., 1996). Other T cell abnormalities in the NOD mouse include defective TCR signaling (Salojin et al., 1997), aberrant responses to superantigens (Radons et al., 1997), and promoter defects in the TAP 1 and LMP 2 genes, resulting in reduced levels of the corresponding proteins, which are involved in antigen processing.

Considering the other side of the equation in NOD diabetes, the ß cell itself and a NOD ß cell line (NIT) have both been reported to have a 2- to 3-fold elevation in free intracellular calcium relative to islets from other strains, suggesting that there are problems in intracellular signaling in this tissue as well (Wang et al., 1996). One factor that <u>can</u> be ruled out as an etiological agent for IDDM in the NOD mouse is complement mediated lysis of the ß cell, since the NOD is C5 negative (Baxter and Cooke, 1993).

Given the chain of events leading to ß cell destruction there are several points along the way where checks and balances would normally be in place to ensure that the immune system responds to infectious threats to the host but not to self-tissues (at least not for any sustained period of time). With the known defects there would seem to be several potential

breaks in the chain of command, beginning with the APCs and leading on to the ultimate effectors, the T cells. This potentially means there are many levels of intervention possible that might stop the chain reaction that autoimmunity seems to represent, and in fact this is the case for many studies with gene knock-out mice, antibody blockade and cytokine administration. However, when considering initial etiological factors responsible for targeting the ß cell (or perhaps the islet) and seeding the insulitis, the Mϕ is a prime suspect. Although this case has been made by Leiter and Serreze for some years (Leiter and Serreze, 1992; Serreze and Leiter, 1988) and additional studies have been carried out examining role of defective APC's in human IDDM (Faustman et al., 1991; Faustman et al., 1992; Pinies et al., 1997), this work has primarily focused on the defective expression of MHC on these cells. Given the endocrine nature of the targets of autoimmunity in the NOD mouse (and human) there may be additional defects in either the APCs (principally dendritic cells) or the target tissue itself that could play a role in the inappropriate homing and activation of the dendritic cells in the environment of the islet. This idea will be developed further in Chapter 5.

Cytokines and immune networks: Cytokines are molecules of low molecular weight (< 30 kD) that function essentially as the "neurotransmitters" of the immune system, although their expression is not limited to cells of the immune system nor are their effects. These molecules are pleiotropic and synergistic and tend to act over short distances. There seems to be an ever growing list of new members to the family of cytokines which can be divided into subfamilies of interleukins (IL), interferons (IFN), tumor growth factors (TGF), tumor necrosis factors (TNF) and colony stimulating factors (CSF). The data base for information relating to cytokines and their properties is so vast and rapidly expanding that the *Encyclopedia of Cytokines* which was originally in print form, is now exclusively in an electronic format to facilitate rapid up dating of the information (www2.lmb.uni-muenchen.de).

In the past decade a paradigm has evolved that integrated some of the dominant regulatory cytokines with the apparently dichotomous relationship between cell mediated immunity and antibody responses that had been observed earlier. In these previous experiments it was demonstrated that over a wide range of antigen doses, as antibody titers increased, cell mediated immunity (as measured as footpad swelling in DTH) would decrease, and vice versa (Parish, 1996). In the cytokine work, it was discovered that murine CD4+ Th clones produced discrete sets of molecules such that Th_1 cells expressed IL-2 , IFNγ and TNFß (lymphotoxin) and Th_2 cells produced IL-4, Il-5, IL-6, IL-10 and IL-13 (Mosmann et al., 1990; Cherwinski et al., 1987). Further, in these cytokine patterns it was recognized that Th_1 factors were associated with cell mediated immunity (e.g. DTH) whereas Th_2 products favored B cell help and antibody production. It was also discovered that these two responses counter balanced each other such that when the immune system was challenged by a pathogen, a choice would have to be made between the two possible cytokine patterns, and the wrong choice could prove fatal (Mosmann and Moore, 1991).

The unifying nature of these concepts, supported as they were by their successful application to animal models of infectious disease (e.g. *Leishmania*), has made the Th_1/Th_2 paradigm a dominant concept in immunology. From its early beginnings with CD4+ Th subsets it has been expanded to include CD8+ CTLs (Tc_1/Tc_2) that parallel the cytokine patterns of their Th counterparts (Mosmann and Sad, 1996). As these horizons have expanded so too has the terminology, and since many of the key regulatory cytokines are made by non-T cells, it has been suggested that cytokines be designated as type 1 or type 2 depending on whether they favor cell mediated or antibody responses (Salgame et al., 1991). In this terminology IFNγ, which is strongly associated with cell mediated immunity, or cytokines which induce IFNγ such as IL-12 or IL-18 are considered type 1, while cytokines such as IL-4, IL-10, and IL-13 are type 2. However, as will be seen in

Chapter 4, the context, timing and dose can all have a profound effect of the immunological outcome when adding any particular cytokine.

Immune modulation in IDDM: The application of type 1 and type 2 principles to autoimmunity needs to be thought of in a different light when considering what in fact is the "appropriate" response. On the one hand, it is clear that the correct response to a self antigen is no response at all rather than type 1 or type 2. However, if an inappropriate choice has been made by the immune system to respond to self (as is the case with autoimmunity) then better to deviate to the least destructive alternative. So what is the preferred (type 1 / type 2) response in the islet?

Much of the IFNγ , IL-4 and IL-10 data that correlates a type 1 response with progression to ß cell destruction and a type 2 response with prevention of disease is presented in Chapter 4, but some recent data for the type 1 cytokines will be described here (reviewed in (Rabinovitch, 1993; Rabinovitch, 1998)). Briefly, in spontaneous disease it can be generally said that increases in type 1 cytokines such as IFNγ (Suarez-Pinzon et al., 1996; Rabinovitch et al., 1995c) and its inducers IL-12, IL-18 (Rothe et al., 1997) correlate with the onset of the destructive phase of insulitis in NOD mice. While disruption of the IFNγ gene caused only a mild reduction of insulitis and a delay in diabetes onset (Hultgren et al., 1996), the disruption of the α chain of the IFNγ receptor resulted in negligible insulitis and no diabetes (Wang et al., 1997). However, complications arise when considering the antibody and cytokine administration data. In studies with exogenous IL-12, depending on how this cytokine was administered it was shown to either reduce diabetes incidence in a dose dependent manner (injections 1 or 3 times a week) (Yang et al., 1994) or accelerate disease onset (daily injections) (Yang et al., 1994; Trembleau et al., 1995). Also, anti-IFNγ can prevent cyclophosphamide induced diabetes (Debray-Sachs et al., 1991), but by administering exogenous IFNγ or a combination of

IFNγ and TNFα (even more effective) insulitis was reduced and development of diabetes delayed (Campbell et al., 1991).

The complex interactions of TNFα in the NOD mouse (as well as in other models such as EAE (Liu et al., 1998)) has been an ongoing saga. Administration of TNFα to 4 week old NOD mice reduced insulitis and the incidence of diabetes (Jacob et al., 1990), but when administered from birth it greatly accelerated the disease (Yang et al., 1994). When expressed as a islet transgene in the NOD, TNFα caused a massive insulitis but blocked progression to disease, and the T cells in these mice were unresponsive to antigen stimulation (Grewal et al., 1996). The blockade at the stage of insulitis was sufficiently robust to withstand repeated cyclophosphamide challenges. These confounding outcomes are though to be mediated through specific populations of dendritic cells or Mϕ's depending on the developmental period and the route of administration. These complexities with TNFα are important to keep in mind when considering the mechanisms involved with certain immuno-stimulants in the NOD, as there is evidence that production of TNFα or other type 1 cytokines by Mϕ's may be involved.

The many faces of tolerance: Tolerance as seen from the T cell's perspective can be thought of in four broad categories: i) deletional tolerance, ii) T cell ignorance, iii) active tolerance or suppression and iv) anergy. The extent to which ii and iii are the same process is not entirely clear. Also, with respect to anergy, some have argued that the term is only appropriately applied to specific IL-2 independent T cell clones that have no relevance beyond the tissue culture dish, (although these clones are very useful for certain in vitro signaling studies). If this view of anergy is correct, the anergic cells are simply "on their way out", and are the by-products of one or another tolerization mechanism. Examples of each of the tolerance categories can be found in the data sets described in this introduction. Deletional tolerance is the most straightforward of the mechanisms and as mentioned above

has now been demonstrated in an islet specific/diabetogenic $CD4^+$ TCR transgenic system (Schmidt et al., 1997). In this case the autoreactive cells bearing the transgenic TCR were deleted when educated in the context of disease resistant I-A molecules (for example $I\text{-}A^b$), whereas they were not by the disease associated $I\text{-}A^{g7}$ molecule. The fact that this deletion occurs at all tells us something about what the transgenic TCR must be capable of responding to. It suggests that either the antigen is present in the thymus as well as in the islet or an alternate peptide is capable of inducing negative selection in the thymus (Riechmann et al., 1988). In another TCR transgenic system it has been demonstrated that if antigens are expressed only in peripheral sites such as the islet, deletional tolerance is not seen (Akkaraju et al., 1997). Thus, for many self-antigens, even in non-autoimmune hosts, central deletion is probably not relevant, and self-reactive clones will exit into the periphery. This moves us on to category ii) and/or category iii) mechanisms of tolerance.

An example of clonal ignorance is provided by the LCMV viral peptide/TCR double transgenic mouse model described in the molecular mimicry section above (Ohashi et al., 1991). In this case although these animals had an enormously high T cell precursor frequency and in vitro the transgenic T cells could kill targets spontaneously, in vivo they would not attack their peptide target and hence were termed ignorant. Whether any "regulation" is required to maintain this state of "ignorance", and what the nature of this regulation might be, is unknown. Because of this uncertainty, this category will not be consider as active suppression. So what then could be considered active suppression?

Two examples of active suppression can be considered, which represent two quite different situations, and presumably only a limited subset of all possibilities. In the NOD class II proinsulin transgenics, the animals were highly resistant to spontaneous disease and did not show T cell proliferation to proinsulin in the absence of priming, but following priming they (unexpectedly) displayed strong responses, equivalent to a non-tolerized OVA

control protein (French et al., 1997). The lack of response without priming could simply reflect a low (i.e. more normal) precursor frequency, and this idea suggests a level of partial deletion, possibly combined with "T cell ignorance" as a consequence of expressing proinsulin on all class II bearing cells. It would seem that regulatory effects must also be invoked here since it is generally agreed that insulin is not the only antigen involved in early insulitis, and therefore tolerance to proinsulin is generalized to other islet antigens (Kaufman et al., 1993).

The other rather unique example of active suppression (and possibly anergy leading to cell death) is exemplified by the NOD transgenic line which expresses TNFα specifically in the ß cells (Grewal et al., 1996). T cells in this model were unresponsive to islet antigens and they were not activated following cyclophosphamide but did form a dense peri-infiltrate around the islet. However, the cells in this peri-infiltrate offered no protection to the ß cells when these mice were challenged with spleen cells from diabetic mice. An important aspect here is that these T cells were not immune deviated toward type 2 responses, they were simply "turned off", and it is not clear what their ultimate fate is in this situation. An additional point to keep in mind is that the immune status of these animals is probably a result of interactions of the transgenically encoded cytokine with the NOD APCs, which as mentioned above are far from normal. The partial normalization of APC function with certain cytokine additions (discussed below) may help explain the perplexing results with certain cytokine treatments or manipulations in NOD mice.

Outside of these forms of deletional and peripheral tolerance (both of which seem defective in the NOD mouse) there is the catch-all of "immune deviation". The list of papers that have found Th_2 deviation to GAD or insulin proteins or peptides is a long one and several of these papers have already been cited. Most frequently in these experiments, delay in or prevention of disease is associated with insulitis, although in some cases the

protection is achieved with little or no insulitis in a percentage of animals (e.g. oral insulin). These manipulations are presumably inducing regulatory cells similar to some of the CD4+ T cells that have been cloned and characterized to date. The interest in these clones is in their ability to induce protection when adoptively transferred into prediabetic mice. It is interesting to note that in contrast to the typical Th_2 deviation reported, these regulatory clones frequently have cross phenotypes, expressing IFNγ, TGFß, and IL-10 or TNFß (Han et al., 1996; Zekzer et al., 1997) and most of the suppressive properties were felt to reside with TGFß. The role of TGFß in CD4 clones capable of down-regulating pathogenic CD4 responses in vivo is not restricted to IDDM models. Exclusive TGFß production (Th_3) was seen in a myelin basic protein (MBP) specific clone which protected against EAE (Chen et al., 1994), and a clone dubbed a "T regulatory cell 1" (Tr1) in a colitis model produced very high levels of IL-10 with smaller amounts of IFNγ and TGFß (Groux et al., 1997). This Tr1 clone had limited proliferative capacity, and both IL-10 and TGFß were required for its regulatory phenotype.

Immuno stimulation in the NOD mouse: Against this back drop of APC defects and immune deviation through cytokine expression, the evaluation of non-specific immune stimulation in the NOD becomes slightly more comprehensible. As reviewed by others (McGregor et al., 1966; Serreze et al., 1989; Takino et al., 1992; Rabinovitch, 1994), a variety of non-specific stimuli have been shown to prevent diabetes in the NOD mouse. These include viruses such as LCMV (Oldstone, 1988), lactate dehydrogenase virus (LDV) (Takei et al., 1992), a viral infection mimic (polyribonucleotide; or Poly [I:C] (Serreze et al., 1989), as well as bacteria and bacterial preparations such as CFA (McInerney et al., 1991; Sadelain et al., 1990; Ulaeto et al., 1992), BCG (Harada et al., 1990), OK-432 (Toyota et al., 1986) and a fungal derivative Ling Zhi-8 (LZ-8) (Miyasaka et al., 1992; Haak-Frendscho et al., 1993). The viruses is this case are not tropic for the ß cell since LCMV infects T cells and LDV is tropic for Mϕ's, but all of the agents induce a strong

immune response and the expression of a variety of cytokines. It is also important to note that to be effective these agents should be administered early and prior to significant insulitis, generally within the first 4 to 6 weeks of life. When administered early, the effect of CFA is dramatic in the NOD with typically 100 % of the mice (60 to 80 % of BB rats) remaining disease free for approximately 150 days, after which a percentage of the animals will become diabetic. However, during this period the "protected" mice are still autoimmune as evident by the pronounced peri-insulitis typically seen in the pancreas, and diabetes can be induced with cyclophosphamide at any time. The mechanisms of protection post-CFA are not hard to rationalize but in reality the complexity of these cytokine networks is immense and most of the details are not known.

A functional assay which perhaps best measures the overall effect of immunostimulation in the NOD is the syngeneic mixed lymphocyte reaction (SLMR), which has a human equivalent known as the autologous MLR (AMLR). This is a measure of the capacity of immune cells to express a regulatory phenotype that will blunt responses to self antigens, and it can be considered as a rough index of levels of peripheral tolerance through "suppressor cell" activity. For example, following poly [I:C] treatment of NOD mice there was a 40 to 70 % increase in the SMLR. Poly [I:C] treatment was also seen to increase LPS stimulated release of IL-1α and IFNα by macrophages. Similarly, *Mycobacterium* are know to induce macrophages to release TNFα and IL-1ß (Mendis and Targett, 1979; Rabinovitch et al., 1995a) and LZ-8 will stimulate PBL cultures to release IL-2, IFNγ , TNFα, IL-1ß and up-regulate ICAM-1, CD11b and CD2 (Miyasaka et al., 1992; Haak-Frendscho et al., 1993). When CFA induced resistance to diabetes was studied in the BB rat, chronic administration of TNFα produced equivalent protection to that seen with a single injection of CFA and the protection was blocked with co-administration of anti-TNF and CFA (Rabinovitch et al., 1995a).

It has been suggested that these immunostimulatory agents (i.e. CFA and BCG) induce a type 2 reaction in the NOD mouse (Shehadeh et al., 1993). However, since IL-4 typically shows little change with CFA administration whereas there is generally a marked reduction in IFNγ in the islet, it may be more appropriate to think of CFA and BCG as partially restoring peripheral tolerance and down regulating inappropriate cell meditated immunity to self rather than up-regulating an equally inappropriate type 2 response.

Objectives and Rationale of This Thesis Project

The experimental work described in this thesis can be divided into two parts. The first part (Chapters 2 and 3) relate to the diabetes autoantigen glutamic acid decarboxylase (GAD) and a potentially cross-reactive *E. coli* protein, and the role that these antigens may play in autoimmunity in NOD mice. The second part of the thesis (Chapter 4) examines the role of type 2 cytokines in the modulation of NOD autoimmune diabetes in a disease recurrence transplant model.

Part 1: ecGAD and IDDM in the NOD mouse:

At the time I began this work the 64 kD islet autoantigen had just been identified as GAD (Baekkeskov et al., 1990) and our collaborator Dr. B Singh had demonstrated that when young NOD mice received a single injection of a partially purified fraction of a bacterial homologue of GAD (*E. coli* GAD or ecGAD), the incidence of diabetes was significantly reduced (B. Singh, unpublished results). The protection observed with ecGAD was comparable to the dramatic results that Dr. Singh and his colleagues had documented with CFA (another bacterial preparation) in the prevention of diabetes in the NOD mouse (Sadelain et al., 1990). These observations raised the question of the potential antigenic relationship between CFA, ecGAD and the mammalian form of the protein. If such a relationship existed, an islet antigen specific response (or at least cross-reactive response) rather than a non-specific immunostimulatory mechanism might be involved with the prevention of diabetes by ecGAD in NOD mice. Since endogenous islet GAD had been identified as a key autoantigen in type 1 diabetes and perhaps central to the etiology of the disease, the role that a potentially cross reactive bacterial form of the protein might play was of great interest. It should be noted that during this time it was also demonstrated that deviation of anti-GAD autoimmunity toward a type 2 response by immunizing young

animals with human GAD65 (Kaufman et al., 1993; Tisch et al., 1993) or murine GAD67 (Elliott et al., 1994) resulted in reduced incidence of disease in NOD mice, and at least with the GAD65 experiments, induced antigen specific hypo-responsiveness.

When we began these investigations the ecGAD gene had not been cloned and only a partial amino sequence was available. Further, protein sequencing was complicated by N-terminal blockage. Hence obtaining the ecGAD DNA sequence was the most direct route to elucidating the complete protein sequence, which we could then compare with the mammalian GAD sequences. In addition to providing the sequence, cloning the ecGAD gene would provide the option of producing recombinant protein. Once the relationship between the *E. coli* and mammalian GAD proteins was known, further studies in the NOD mouse with ecGAD were performed to evaluate the likelihood of an antigen specific effect on disease prevention.

Part 2: adenovirus expression of IL-4 and IL-10 in NOD islets:

In the context of islet transplantation into diabetic patients, in general the recurrence of autoimmunity is a greater barrier to success than is allograft rejection. In fact, the immunosuppressive drugs used to control allorejection generally do not prevent recurrent autoimmune destruction of the transplanted ß cells. Over the past few years evidence has accumulated that type 1 diabetes is a T cell mediated disease, and that the cellular infiltrate that is responsible for ß cell destruction expresses type 1 cytokines (Rabinovitch, 1998). Experiments in the NOD spontaneous disease model demonstrated that IL-4 or IL-10 could prevent diabetes, which provided support for the Th_1/Th_2 paradigm in IDDM. Also, when infiltrates in sentinel islet grafts were studied in diabetic NOD mice which were treated with CFA or with saline at the time of islet transplant, type 1 cytokines (i.e. IFNγ) expressed in the islet graft were associated with recurrence of diabetes in the saline group, and type 2 cytokines were found in the grafts of CFA treated animals where diabetes did not recur.

Further, collaborators here at the University of Alberta demonstrated that systemic IL-4 and IL-10 administration delayed disease recurrence in the diabetic NOD/syngeneic islet transplant model, although the prolongation of graft survival was relatively modest (Rabinovitch et al., 1995b). We hypothesized that these type 2 cytokines would be more effective if they were secreted locally in the vicinity of the islets. We therefore undertook experiments to examine this possibility using recombinant adenovirus transfection technology, since these viruses had been shown to give very efficient gene transfer in a rat islet model.

Since recombinant adenoviruses had not been used with mouse islets before, the first objective was to determine if mouse islets could be transduced using a reporter virus. Once it was established that adenoviruses were an appropriate vector for our purposes, mouse IL-4 and mouse IL-10 adenoviral constructs were engineered and recombinant viruses created. Expression of the relevant cytokines by our recombinant viruses was confirmed by transducing mouse islets and assaying the supernatants. Since it was unclear what effect the expression of IL-4 or IL-10 might have on islet function, this was also examined *in vitro*. Concurrent with these experiments were in vivo experiments involving transplantation of islets transduced with a reporter virus into non-autoimmune Balb/c mice. This allowed us to determine how long expression of the exogenous virally encoded reporter protein would occur in the grafts in the absence of any anti-islet immune responses. The final step of this project involved a direct test of the cytokine constructs in transduced islets transplanted into diabetic NOD recipients.

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Chapter 2

Escherichia coli Has Two Homologous Glutamate Decarboxylase (*gad*) Genes That Map To Distinct Loci

A version of this chapter has been published.

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Introduction

The enzyme glutamate decarboxylase (GAD; also known as glutamic acid decarboxylase; EC 4.1.1.15) catalyzes the α-decarboxlyation of glutamic acid to produce γ-amino-butyric acid (Fig. 2-1A). Within bacteria, GAD activity seems to be relatively unique to *Escherichia coli* (Schubert et al., 1988). Gale (Gale, 1946) proposed a general role for the inducible bacterial amino acid decarboxylases, including GAD, in the maintenance of physiological pH under acidic conditions. *E. coli* GAD (ecGAD) has been extensively characterized with respect to its biophysical and biochemical properties (Gale, 1946; Shukuya and Schwert, 1960c; Shukuya and Schwert, 1960b; Shukuya and Schwert, 1960a; Tikhonenko et al., 1968; Strausbauch and Fischer, 1970a; Strausbauch and Fischer, 1970b; Sukhareva et al., 1979; Morosov et al., 1982; Almasov et al., 1985; Mishin and Sukareva, 1986; Schubert et al., 1988; Sukhareva et al., 1989) and a partial protein sequence has been available for some time (Strausbauch and Fischer, 1970a; Strausbauch and Fischer, 1970b). Based on genetic linkage studies in *E. coli*, the structural gene encoding ecGAD (*gadS*) and a potential regulatory gene (*gadR*) have been mapped between *mtl* at approximately 80.7 minutes and *gltS* at approximately 82.4 minutes (Bachmann, 1990; Lupo and Halpern, 1970; Marcus and Halpern, 1969; Marcus and Halpern, 1967), but these early results have not been followed up. For the purposes of our

immunological studies, we were interested in obtaining the complete DNA and protein sequence of ecGAD. At the time this work was initiated, an extensive search of the literature and all available sequence data bases suggested that neither the complete protein sequence nor the gene sequence for ecGAD had been determined. To obtain this information, we generated a DNA probe for the gene based on a recent more extensive partial protein sequence (Maras et al., 1990). By using this probe on *E. coli* genomic DNA, we discovered that there were not one but two separate cross-hybridizing *gad* genes. We report here the complete DNA sequences and map positions for these two genes, which encode two distinct ecGAD polypeptides. The genes are highly similar, yet they map to separate loci, neither of which correspond to the previously reported map position for *gadS*. Representatives from a diverse assemblage of *E. coli* strains were examined, and all were found to contain two *gad* genes. The conservation of two homologous coding segments among diverse strains of *E. coli* suggests that both gene products play a role in *E. coli* metabolism. Although the complete protein sequence of one isoform of ecGAD was reported while this manuscript was in preparation (Maras et al., 1992), this is the first account of the dimorphic nature of the *gad* genes in *E. coli*, and the first time the complete DNA sequence of either isoform has been reported.

Methods and Materials

Culture media, bacterial strains and bacteriophage libraries: All bacterial cultures were grown in 2xYT (36) at 37°C with vigorous aeration. Recombinant plasmids were propagated in standard *E. coli* DH5α, WM1100, or TG2 as required. The Kohara λ EMBL4 'miniset' library (version 9010) was obtained from the Japanese National Institute of Genetics (Kohara et al., 1987) and plated on *E. coli* NM 621. All other strains used are described in Table 2-2, with the exception of *Salmonella typhimurium* LT2 (Zinder and Lederberg, 1952).

General Procedures: To obtain *E. coli* DNA, we lysed cells by re-suspending the drained bacterial pellets in 100 mM Tris-HCl- 25 mM EDTA-1% sodium dodecyl sulfate (SDS) and incubating them for 15 min. at room temperature. The lysate was extracted with phenol, phenol-chloroform and chloroform, and the DNA was precipitated with isopropanol, rinsed in 70% ethanol, and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). To obtain λ phage DNA, we purified the intact phage particles on CsCl step gradients and extracted the DNA as described in reference 36. To generate the *gad* gene probe, polymerase chain reaction (PCR) conditions were 94, 55, and 72°C, each for 1 minute, in a total reaction volume of 100 µl. Buffer, primer, nucleotide, and *Taq* DNA polymerase concentrations were as recommended by the supplier (Perkin-Elmer Cetus, Norwalk, Conn.). For each PCR, 5 ng of purified *E. coli* DNA was amplified through 30 cycles, and the products were analyzed directly on a 1.2% low melting point (LMP) 1.2% agarose gel. The sequences of the oligonucleotide primers used in the PCR were are shown in Fig. 2-1B. For hybridization probes, DNA fragments were excised from LMP agarose gels, boiled, and labeled with α^{32}P-dCTP by the random hexamer primer method (Feinberg and Vogelstein, 1983). Southern blots were transferred to High Bond-N (Amersham Corp., Arlington Heights, Ill.) and probed and washed as described previously (Elliott et al.,

1990). Southern blots of *S. typhimurium* DNA were probed at a lower stringency (5 x SSC [1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS, 5 x Denhardt's solution at 37°C) and washed in 5 x SSC-0.1% SDS at 56°C. DNA sequencing was done on double-stranded and single-stranded templates with the Sequenase Kit (United States Biochemical, Cleveland, Ohio) and a series of synthetic oligonucleotide primers. Searches for homologous sequences were run for both DNA and encoded protein against the GenBank, Unique EMBL and HIV DNA or the PIR, SWISS-PROT, and HIV protein data bases (Release 92.1.1; Intelligenetics, Mountain View, Calif.). The searches were conducted by using FastDB from the Intelligenetics Suite (Release 5.4) on a Sun SPARC Station 2 (Sun Microsystems Inc., Mountain View, Calif.).

Construction and screening of plasmid libraries: The cloning vector pHAS (Fig. 2-2A) was constructed by modifying the vector pBluescript KS-(Stratagene, La Jolla, Calif.) in the following way: i) digestion of KS- with *Bst*XI, blunting with T4 DNA polymerase, and re-ligation to delete the *Bst*XI and *SacII* sites present in the original polylinker, ii) digestion of the resultant plasmid with *Eco*RV and ligation to the annealed oligonucleotides (5' pCCACGTGTTTGGTGTG 3'- 5' pCCAAACACGTGG 3'), and iii) ligation of the resultant linear plasmid to the ≈360 bp *Bst*XI fragment from the vector pCDM8 (Seed, 1987) to create a circular molecule, pHAS. For construction of libraries, pHAS DNA was digested with *Bst*XI, the ≈380 bp DNA stuffer fragment removed by running the cut vector DNA twice on an LMP 0.8% agarose gel, and the DNA was purified from the final gel sliced by using GeneClean (Bio 101, La Jolla, Calif.).

Two separate *Eco*RI libraries were constructed by digesting 10 µg of *E. coli* DNA with *Eco*RI, size fractionating the digested material on an LMP 0.7% agarose gel, excising gel slices containing fragments of 4.4 kb to 6.6 kb and 9.4 kb to 23 kb, and extracting the DNA from each slice with GeneClean. The DNA fragments were ligated in separate

reactions to a 50-fold molar excess of the oligonucleotides (5' pCTCTAGGG 3'-5' pAATTCCCTAGAGACAC 3'), purified again by electrophoresis on LMP agarose gels and GeneClean, and ligated into pHAS prepared as described above. A single *Hin*fI library was constructed in the same manner by digesting *E. coli* DNA with *Hin*fI, size fractionating the digested material on a 1.0 % LMP agarose gel, excising a single gel slice containing fragments of 1.2 to 2.3 kb, and ligating to a 50-fold molar excess of the oligonucleotides (5' pCTCTAGGG 3'- 5' pANTCCCTAGAGACAC 3'; where N represents equal portions of G, A, T and C). The final ligations of *E. coli* DNA into pHAS were precipitated with sodium acetate-ethánol, using 10 µg of yeast tRNA (GIBCO BRL, Gaithersburg, Md.) as carrier, transformed into *E. coli* WM1100 by electroporation (Dower et al., 1988), and plated on ampicillin plates. Colonies were transferred to nitrocellulose filters (Schliecher & Schuell,Inc., Keene, N.H.) and the filters were baked and prewashed (Sambrook et al., 1989) and then hybridized and washed under the same conditions as for Southern blots.

Plating and probing the Kohara miniset library: See additional methods section in Appendix 1.

Nucleotide sequence accession numbers. The DNA sequences reported here have been submitted to GenBank under accession numbers M84024 and M84025.

Results

Four oligonucleotides were designed based on the partial protein sequence of ecGAD (Maras et al., 1990), using the regions of protein sequence shown in Fig. 2-1 and underlined in Fig. 2-8. In four separate reactions, all possible combinations of 5' and 3' primers were amplified through 30 cycles of PCR. Four control reactions, each containing *E. coli* DNA plus a single primer, were done in parallel. All the PCRs which contained two primers generated a single predominant fragment of approximately 470 bp, whereas the control reactions did not generate DNA fragments of any length Fig. 2-4. The four amplified products were excised from the gel, and the DNA sequence of each was determined on both strands by direct sequencing by using the PCR primers. Each of the DNA sequences encoded a single long open reading frame, which when translated corresponded to the known partial protein sequence of ecGAD (Maras et al., 1990). One of the DNA fragments was radiolabeled and used as a hybridization probe for the *gad* gene.

We digested *E. coli* DNA from a K-12 strain (J53-1) with the restriction enzymes *Eco*RI, *Hin*dIII, *Hin*fI, *Pst*I and *Sma*I and made a Southern blot. When the blot was probed with the *gad* PCR gene fragment, we found two cross-hybridizing bands of different sizes but roughly equal intensities in all of the digests except *Pst*I. The single band seen with the *Pst*I digest was approximately double the intensity of the bands produced in the other digests. Representative results for the *Eco*RI, *Hin*fI, and *Pst*I digests are shown in Fig. 2-5. With the exception of *Pst*I, we knew from the DNA sequence that our gene probe did not contain any of these restriction sites. These results indicated that there must be two cross-hybridizing genes in *E. coli*. Moreover, the double intensity *Pst*I band suggested that this enzyme cleaves the same-sized fragment from each of the two genes. Although the probe does contain an internal *Pst*I site, it is so near one end that the PCR product apparently does not hybridize efficiently to any adjacent *Pst*I fragments under

these stringency conditions. Both the 13.1 kb and 5.6 kb *Eco*RI fragments, and the 2.2 and 1.65 kb *Hinf* I fragments shown in Fig. 2-5 were chosen for further analysis.

Clones '*Eco*RI L' (13.1 kb *Eco*RI fragment) and '*Eco*RI S' (5.6 kb *Eco*RI fragment) were isolated from separate libraries. A third library which contained both the longer and shorter cross-hybridizing *Hin*fI fragments was constructed and screened, and clones were isolated and determined to contain either the 2.2-kb ('*Hin*fI L') or 1.6 kb ('*Hin*fI S') fragment on the basis of restriction mapping (Fig. 2-6). Sequence data obtained by primer walking through the cloned genomic fragments revealed that although each contained an open reading frame, no single clone encoded a complete *gad* gene. However, since the DNA sequence of the two *gad* genes is similar but not identical (see below), by comparing sequences of the four clones, we were able determine that the *Eco*RI L clone should be matched with the *Hinf* I S clone, and vice versa as shown in Fig. 2-7 (upper parts). By combining the DNA sequences from the matched pairs of clones, we were able to reconstruct the entire open reading frame for each gene.

The aligned DNA and encoded protein sequences of *gadA* and *gadB* are shown in Fig. 2-8. Each gene contains a long open reading frame which encodes a protein of 466 amino acids, with calculated molecular masses of 52,651 and 52,634 D respectively. A putative ribosome binding sequence is found immediately 5' of the start codon (underlined in Fig. 2-8). Preliminary sequence data (not shown) suggest that there are potential promoter sequences (Hawley and McClure, 1983) 5' to each of the *gad* genes which implies they are both functional genes. The two DNA coding sequences are 98% similar, and the deduced protein sequences show 99% similarity. The 5 amino acids which differ between the two proteins all occur within the N-terminal 22 residues, and some of these represent non-conservative substitutions (Fig. 2-8 and Table 2-1, upper part). Our deduced protein sequence for ecGAD α is in agreement with the published protein and deduced protein

sequences for ecGAD (Maras et al., 1992), with the differences listed in the lower part of Table 2-1. The *E. coli gad* gene sequences reported here were not obtained from PCR amplification, which can generate mutations (Lundberg et al., 1991), and for each *gad* gene, we independently sequenced the upper and lower strands and found the two strands to be in complete agreement with each other. We suggest therefore that our DNA and deduced protein sequences are correct and that those differences which exist between our sequences and the protein and partial DNA sequences published by Maras *et. al.* (1992) are due either to polymorphisms between the different strains of *E. coli* studied or to errors in the previously published sequences, which in the case of the DNA data may have resulted from artifacts introduced during PCR amplification.

gadA and gadB chromosomal localization: To map the positions of the two *E. coli gad* genes, we radio-labeled the *Hin*fI L and *Hin*fI S DNA fragments and in separate reactions hybridized these to duplicate sets of filters containing DNA from the Kohara miniset library (Kohara et al., 1987). With both probes, three of the λ EMBL 4 clones gave a strong hybridization signal [clones 10C7(279), 22E3(280) and 9G3(606)], with the stronger signal for the *Hin*fI L probe corresponding to clone 9G3 and the stronger signal for the *Hin*fI S probe corresponding to clones 10C7 and 22E3. At least five additional λ clones gave an intermediate cross-hybridizing signal that was clearly below that of the 9G3, 10C7, and 22E3 clones but above background (discussed below). Knowledge of the map positions of the strongly cross-hybridizing λ clones together with the lengths of the two *Eco*RI fragments enabled us to assign positions for *Eco*RI L and *Eco*RI S and to deduce the orientation of the open reading frames for the two *gad* genes (Figs. 2-7 and 2-8). To confirm the assignments for the *Hin*fI L and *Hin*fI S fragments, we first purified λ DNA from the 10C7, 22E3, and 9G3 phages, digested it with *Hin*fI, made Southern blots, and then probed with the original *gad* gene probe (Fig. 2-5., right lanes). These results indicate clearly that clone 9G3 contains the *Hin*fI L fragment, and clone 22E3 contains the *Hin*fI S

fragment. Clone 10C7 appears to contain a slightly truncated *Hin*fI S fragment, suggesting that it is missing a portion of the *gad* gene, a result which is in keeping with our understanding of the map position of this λ clone relative to the position we assigned to *gadB* (Fig. 2-7). The miniset library was constructed from *Sau*3AI partial digests of *E. coli* W3110 DNA, and examination of the DNA sequence for *gadB* reveals several *Sau3*AI sites downstream of the two *Hin*fI sites (Figs. 2-7 and 2-8). Therefore, the insert in clone 10C7(279) was apparently generated by digestion of the *E. coli* chromosome at one of these *Sau*3AI sites, and the truncated *Hin*fI fragment results from cutting at the usual *Hin*fI site on the one side, and a *Hin*fI site present in the λ vector on the other. With respect to the *E. coli* physical map, there is an inversion in the chromosome of *E. coli* W3110 in the region that encompasses *gad* α (Hill and Hamnish, 1981). Thus, although the two *gad* genes are shown to be transcribed in opposite directions in Fig. 2-7, this would not be the case in other *E. coli* K-12 strains.

All of our initial results for the *gad* gene were based on *E. coli* K-12 strains. We were interested in knowing whether other strains of *E. coli* also had two *gad* genes, or whether this was a property which was unique to the K-12 strains. The ECOR collection of *E. coli* strains provides a set of bacteria with diverse genetic backgrounds, originating from a wide range of hosts (Ochman and Selander, 1984). We extracted DNA from a number of representative members in this collection (Miller and Hartl, 1986), digested each with a series of restriction enzymes, and subjected these fragments to Southern blot analysis using a *gad* gene probe. The results of these experiments are summarized in Table 2-2. Although the size of the cross-hybridizing bands varied somewhat between strains, all *E. coli* strains examined contained two bands for most digests. It would appear, therefore, that most if not all strains of *E. coli* contain two *gad* genes.

Since *S.typhimurium* is closely related to *E. coli*, we wondered whether we could detect a cross-hybridizing *gad* gene in this species. Even at the lowest stringency of hybridization, we were unable to detect a clear signal on Southern blots made from *S. typhimurium* DNA using the *E. coli* gene as a probe (data not shown). Very long autoradiographic exposures revealed multiple faint cross-hybridizing bands, but these were more consistent with the less specific signals seen with long exposures of the *E. coli* Southern blots. If in fact *S. typhimurium* does make GAD, we suggest the gene sequence is significantly different from that found in *E. coli*.

Discussion

To obtain a gene probe for *E. coli gad*, we designed oligonucleotides based on two independent segments of protein sequence whose relative positions were known and then used PCR to generate the unknown gene sequence lying between them. This represents a powerful application of the PCR technology which is far superior to screening strategies based on degenerate oligonucleotides. To clone the *E. coli gad* genes quickly and efficiently, we constructed a series of sub-libraries by digesting *E. coli* DNA with *Eco*RI or with *Hin*fI and selecting fragments within a certain size range as described in the Materials and Methods. To facilitate the construction of the sub-libraries, we created a new plasmid vector, pHAS. When pHAS DNA is digested to completion with *Bst*XI, a DNA fragment of approximately 380 bp is cleaved from the center of the polylinker, and the resulting ends of the vector are sticky but not self-cohesive (Seed, 1987). Ligation of foreign DNA into *Bst*XI cut pHAS prepared as described in the Materials and Methods is highly efficient and results in very low numbers of non-recombinant (i.e., empty) clones in the final libraries, typically < 0.1%.

Since our deduced ecGAD protein sequences are virtually identical to the recently published protein sequence of Maras *et al.* (1992), we are confident that the *gadA* and *gadB* sequences shown here do indeed encode *ecGAD*. Further, all three protein sequences are in agreement with the early partial protein sequence and total amino acid composition data (Strausbauch and Fischer, 1970a; Strausbauch and Fischer, 1970b). Predicted molecular masses for both isoforms of ecGAD are also in agreement with estimates based on sedimentation rates for monomeric ecGAD protein (Strausbauch and Fischer, 1970a). Protein sequence homology searches conducted by using the FastDB algorithm produced results similar to those obtained by Maras and colleagues (Maras et al., 1992). Our optimized searches identified a plant tryptophan decarboxylase (de Luca et al., 1989) as the

most similar protein in the data bases. However, when our ecGAD protein sequences were aligned with other protein sequences from the family of pyridoxal-dependent decarboxylases (aligning with respect to the active site lysine which binds pyridoxal phosphate), the *E. coli* protein sequences contained many of the conserved residues which were originally noted by Jackson (Jackson, 1990) for this group of enzymes. For the ecGAD α and ecGAD β proteins reported here, the region containing these conserved residues, which likely contributes to the active site, extends from the methionine at position 240 to the cysteine at position 284 (data not shown). Very little protein sequence similarity exists outside of this region when comparing the *E. coli* protein sequences with the known eukaryotic GADs (see Chapter III), and this observation also extends to other members of the group of pyridoxal-dependent decarboxylases, both eukaryotic and prokaryotic. It is interesting that in those eukaryotic organisms in which the *gad* genes have been cloned, two related but non-cross-hybridizing genes have virtually always been found (Bu et al., 1992).

As noted in the Results, longer exposures of both the *E. coli* and *S. typhimurium* Southern blots allowed us to detect numerous additional weakly cross-hybridizing bands. These likely represent genes encoding other pyridoxal-dependent decarboxylases, since our probe included the highly conserved region bracketing the pyridoxal binding lysine. Such an explanation would also account for the intermediate hybridization signals observed for some of the clones when the minset library was screened.

Our hybridization results with the miniset library, in combination with the restriction mapping data and Southern blot analysis, argue strongly that we have correctly assigned the positions of the two *gad* structural genes (*gadA* and *gadB*) on the *E. coli* physical map (Fig. 2-7). The fact that neither *gad* gene mapped to the 82 minute region, assigned to *gadRS* (Bachmann, 1990), suggests that this assignment should be revised, although it is

possible that a regulatory gene (*gadR*) accounted for the earlier mapping results. It is also possible that a single copy structural or regulatory gene responsible for the production of the pyridoxal phosphate cofactor could map to the 82 minute position. A mutation at such a locus could result in a loss of GAD activity, as well as a loss of enzyme activity for all other pyridoxal-dependent enzymes. This type of mutation, however, would not necessarily affect the production of pyridoxal-dependent enzymes. GAD immunoreactive protein with little or no enzymatic activity was observed by Lupo and Halpern (Lupo and Halpern, 1970) in a *gad*$^-$ *E. coli* strain , but this was interpreted to represent a lesion in the *gad* structural gene and considered supportive of the 82-minute map position with a single *gad* locus.

The similarity between the two *gad* genes and the encoded proteins is striking. Other examples of duplication of structural genes in which the two homologs map to distinct loci in *E. coli* include *arg*I and *arg*F (Bencini et al., 1983; Van Vliet et al., 1984) and *tuf*A and *tuf*B (An and Friesen, 1980; Yokota et al., 1980). The *arg* genes have diverged to the point that the two enzymes are biochemically distinguishable (Legrain et al., 1976; An and Friesen, 1980). In contrast, the *tuf* genes are nearly identical, and the only difference in the 339 residue structural proteins is a single substitution at the C terminus. The *tufA* and *tufB* gene products are essentially indistinguishable functionally and structurally (Furano, 1977; Miller et al., 1978), but the Tuf*A* protein appears to be produced at higher levels (Pedersen et al., 1976a; Pedersen et al., 1976b). Comparison of the *tufA* and *tufB* gene sequences shows that nucleotide differences tend to be clustered toward the 5' and 3' ends of the genes, with very few differences occurring in the central region. This same clustering of differences is also observed in *gadA* and *gadB* (Fig. 2-8). An and Friensen (1980) suggested that in the case of *tufA* and *tufB* this clustering of differences is consistent with the notion that recombination events are more likely to occur within the central portion of the genes. Alternatively, it can be argued that physiological constraints

have prevented divergence of these homologous gene pairs. Since the gene products of the *tuf* loci were functionally indistinguishable, it was suggested that the duplication provided an emergency backup when demand for translation elongation factor EF-Tu was high (An and Friesen, 1980). A similar argument may apply to the two *gad* loci, since the two ecGAD proteins are virtually identical. Beyond this consideration how else might two such similar genes serve *E. coli*?

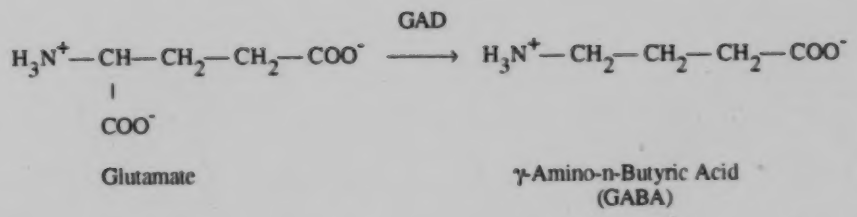
Although our work does not address this question directly, clues may be found in earlier biochemical studies by Sukhareva and colleagues (Sukhareva et al., 1979; Tikhonenko et al., 1968). The functional ecGAD enzyme is a hexamer composed of three dimers, with each monomer containing a single pyridoxal phosphate molecule. The dimer to hexamer transition is pH dependent and requires pyridoxal phosphate. In the presence of coenzyme, hexamers form at pH 4.6, and if the coenzyme is then removed the hexamers will dissociate at pH 6.0. This conversion of dimers to hexamers with the addition of pyridoxal phosphate is linked to reversible changes in the number of exposed cysteine residues (Sukhareva et al., 1989). The dimer, on the other hand, is extremely stable and requires guanidine-HCl or SDS to dissociate the complex (Sukhareva et al., 1979). To date it has generally been assumed that *E. coli* had a single ecGAD enzyme that was composed of six identical monomers. However, Strausbach and Fisher (Strausbauch and Fischer, 1970a) allowed for other possibilities with their conclusion that the functional protein was composed of "identical or nearly identical subunits". It is possible that the hexmer is composed of hetero or distinct homo-dimers, or perhaps a combination of the two. An alternate view would be that each GAD hexamer is composed of identical subunits and that two types of ecGAD exist that differ in their functional properties. Some unique properties of ecGAD relative to other decarboxylases in *E. coli* (discussed below) may favour the later position. With the cloning of the full length genes, resolution of this question is now possible since the genes can be expressed independently and the proteins

subject to functional studies. The possibility that this duplication is of no functional consequence is unlikely, given the maintenance of two genes in diverse strains (Table 2). This latter point in conjunction with the fact that GAD activity is ubiquitous amongst *E. coli* strains but far less common in other *Enterobacteraceae*, and undetected in a large number of *Pseudomonadaceae*, *Aeromonadaceae* and *Streptococci* strains examined (Gale, 1946; Schubert et al., 1988) argues for a relatively unique role for GAD in *E. coli*.

Gale (Gale, 1946) proposes a role for ecGAD in the maintenance of physiological pH under acidic conditions. In this context, ecGAD is only one of a variety of inducible bacterial decarboxylases which can accomplish this purpose. However, among this group ecGAD is unique in that although ecGAD activity is enhanced by the presence of its substrate (glutamate) as well as by low pH (4.0-4.5), there is also a low level constitutive expression of the enzyme at higher pH (8.5) and in the absence of substrate (Gale, 1946). All decarboxylases will tend to neutralize the micro environment as a result of the liberation of CO_2 from substrate, and no doubt this is an essential function for ecGAD. If one considers the possible consequences of ecGAD activity that transcend the immediate physiological requirements of *E. coli*, one cannot fail to notice that the product of ecGAD activity is the eukaryotic neurotransmitter γ-amino-butyric acid (GABA). Although we are unaware of any reports indicating GABA release from *E. coli*, or of increases in ecGAD activity associated with pathogenic infections, Gale (Gale, 1946) does report a large increase in activity of a pH inducible tyrosine decarboxylase from isolates of *Streptococci faecalis* obtained from infants with diarrhea. Considering that any bacterial overgrowth tends to produce an acidic micro environment, it is intriguing to consider the possibility that under such conditions *E. coli might* interact with host tissues via a GABA signaling mechanism.

Speculation aside, the availability of the cloned *gad* genes together with accurate information about the location of *gadA* and *gadB* on the *E. coli* chromosome should facilitate a genetic approach to elucidate the role of GAD in *E. coli* metabolism.

A



B

5' Primers

		134	135	136	137	138	139	
Amino Acid Sequence		Gly	Met	Ala	Met	Lys	Trp	
Primers B GAD 6a	5'	GGN	ATG	GCN	ATG	AAA	TGG	3'
B GAD 6b	5'	GGN	ATG	GCN	ATG	AAA/G	TGG	3'

3' Primers

		284	285	286	287	288	289	
Amino Acid Sequence		Cys	Gly	Trp	Val	Ile	Trp	
Upper Strand DNA	5'	TGC/T	GGN	TGG	GTN	ATC	TGG	3'
Primers B GAD 7a	3'	ACG	CCN	ACC	CAN	TAG	ACC	5'
B GAD 7b	3'	ACG/A	CCN	ACC	CAN	TAG	ACC	5'

FIG. 2-1. The molecular "prey" and its "predators". The enzymatic activity of glutamic acid decarboxylase (GAD; A) and the degenerate DNA primers used during initial PCR to synthesize the 470 bp *E. coli gad* probe (B). The *gad* primers were based on the extensive partial amino acid sequence of GAD (Maras et al., 1990), where the numbers above the amino acid residues correspond to their position in the full protein sequence (Fig. 2-8). Primers were selected to generate a convenient size probe in a region of low codon redundancy. Positions in the primers where two DNA nucleotides are separated by a forward slash indicate two possible bases at that position and N implies any one of the four bases could be present in a given primer.

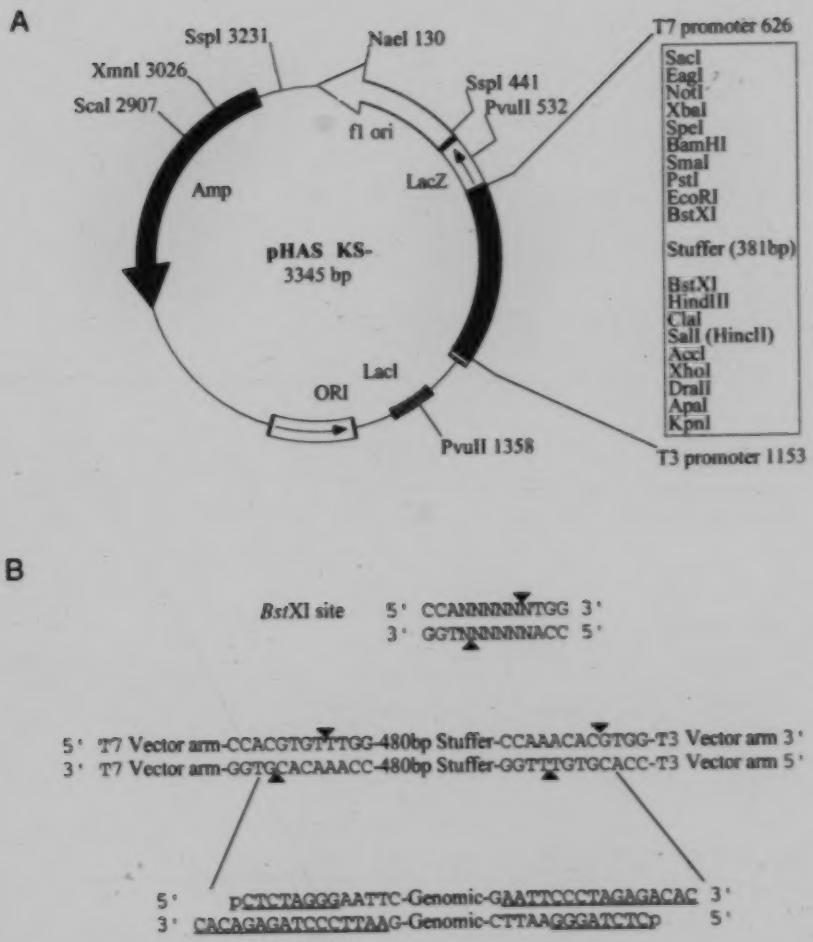


FIG. 2-2. Schematic representation of pHAS cloning vector used for *Bst*XI cloning strategy of *E. coli gad* genes. This vector contains two *Bst*XI sites that flank a "stuffer region" (A) which is replaced by linkered target DNA during the cloning. The *Bst*XI cloning strategy takes advantage of the six N degenerate base positions within the recognition site of this restriction enzyme (B). This degeneracy permits the engineering of over hangs within the sites such that they are complementary in anti-parallel. Therefore, when the vector is digested with *Bst*XI, the overhangs at either end of the vector arms will not hybridize unless provided with an appropriate linker, or linkered genomic DNA in this case. The example shown in the schematic (B) illustrates *Eco*RI digested DNA that has been ligated to such a linker (underlined sequence). In turn, the linkered inserts can hybridize to the *Bst*XI digested vector and with the addition to ligase the plasmid would re-circularize. One other advantage of this polylinker design is that the stuffer provides a diagnostic tool which indicates that both *Bst*XI sites on the vector have been cleaved. Such dual cleavage results in the release of the 360 bp suffer which is easily resolved on an agarose gel (Fig. 2-3).

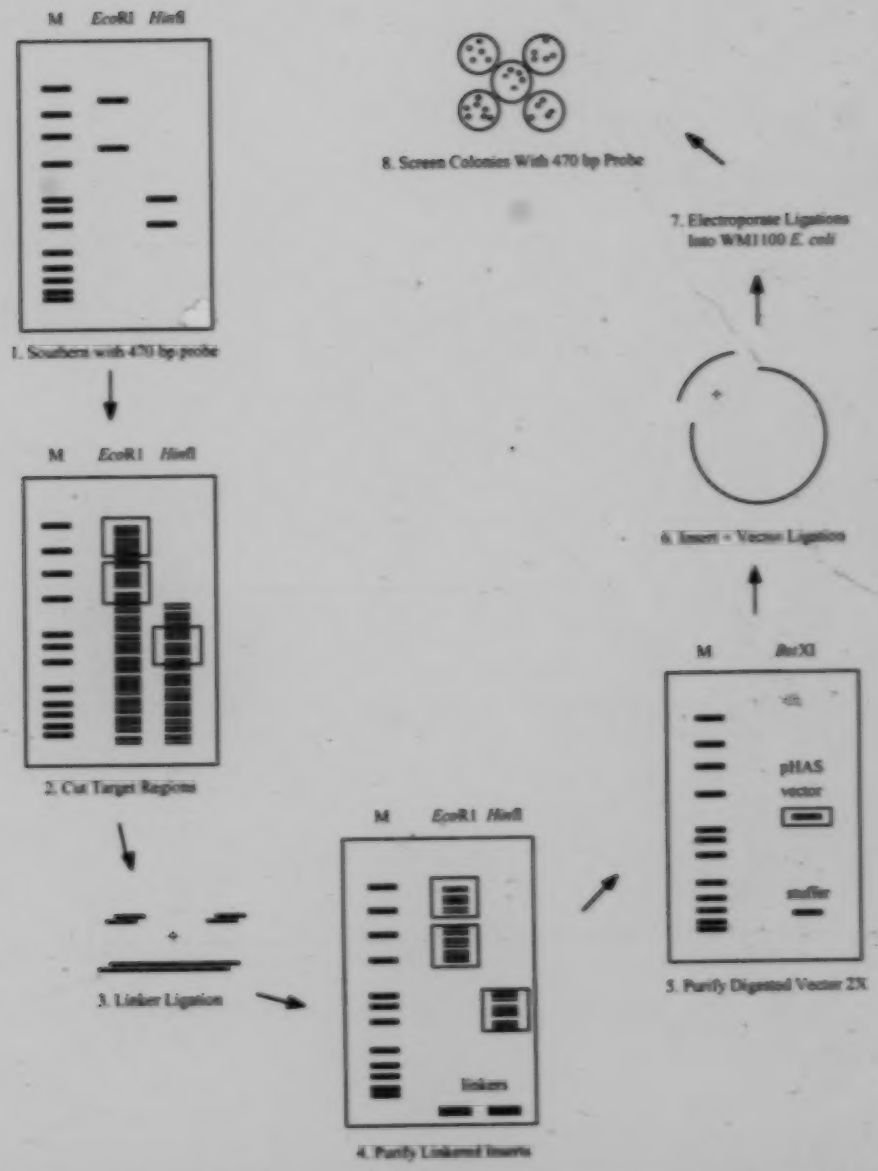


FIG. 2-3. Cloning of *E. coli gad*A/B genomic sequences using a 470 bp PCR *gad* sequence probe. Southern blots revealed two cross hybridizing bands for several restriction digests including *Eco*RI and *Hin*fI, which are illustrated in this schematic. The strategy shown highlights the size restricted libraries that were engineered to minimize the screening and maximize the chances of isolating clones corresponding to the bands identified on the Southern blots (1). Boxes on panels 2, 4, and 5 represent the areas of agarose gel cut to isolate DNA of the appropriate size, which was subsequently separated from the gel with GeneClean to enhance ligation efficiency. The *Bst*XI digested vector was purified twice to reduce background caused by the stuffer. Details are provided in the Methods and Materials.

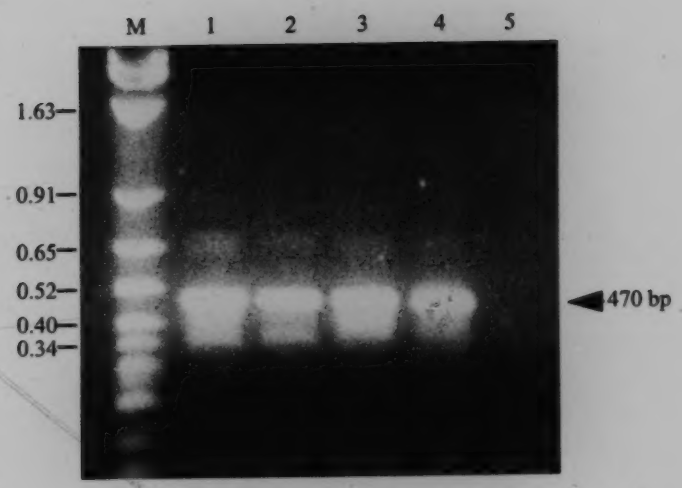


FIG. 2-4. Predicted PCR product with each of the combinations of degenerate primers. Lanes 1 to 4 represent the following pairs of primers (Fig. 2-1); 1) B GAD 6a and 7a, 2) 6a and 7b, 3) 6b and 7b, 4) 6b and 7b, and lane 5 is one of the four control lanes each of which had a single primer. The markers on this 1.2 % TBE agarose gel are pBR322 *Eco*RI/*Eco*RI+PvuII and *Alu* / *Hin*fI respectively and are indicated in kb. Due to the high concentration of agarose, the bands above 1.63 kb are less useful and are not marked.

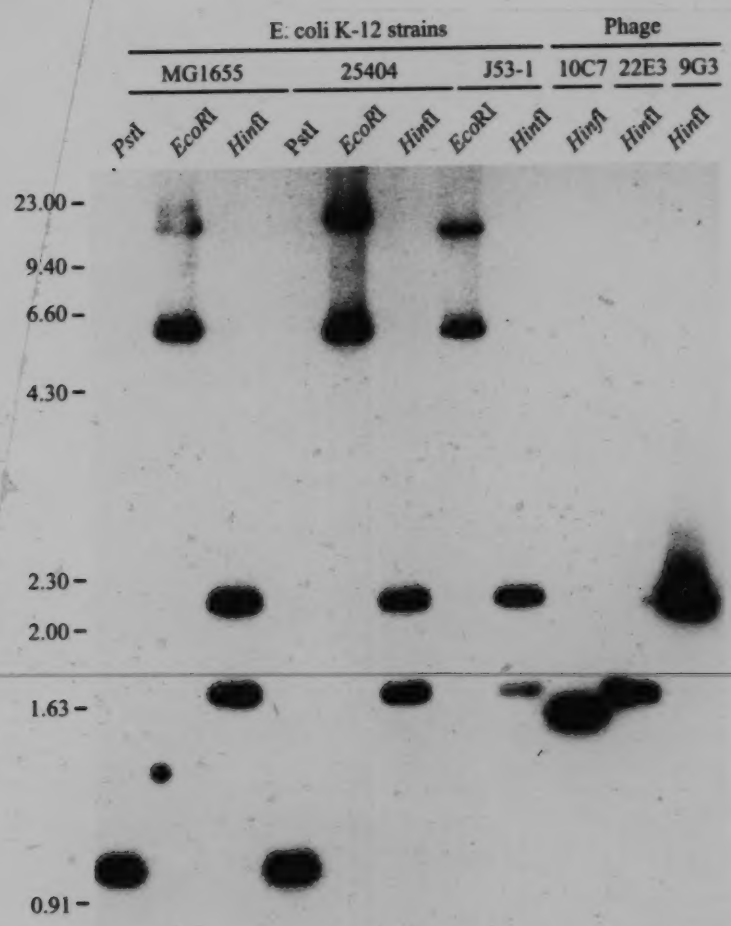


FIG. 2-5. Representative Southern blot made with *E. coli* DNA from three K-12 strains (left eight lanes) and with purified λ phage DNA from three of the clones in the miniset library (right three lanes), digested with enzymes as shown. The blot was probed with a ^{32}P-labeled 552-bp *Pst*I-*Eco*RI restriction fragment from *gadA*. A single band of approximately double intensity appears in the *Pst*I digests because the two genes contain identical *Pst*I digestion fragments (see Fig. 2-7 and 2-8). Numbers on left show size in kilobases.

FIG. 2-6. *E. coli gad* clones isolated from genomic libraries. A total of 32 positive colonies were isolated and of these, 2 clones were selected from each library for characterization. The 6 clones were mapped with a *Xho*I/ *Xba*I restriction digest to release full inserts as shown. Each of the 4 target sequences was obtained in this first iteration. The arrow identifies the linearized pHAS vector and the size markers are indicated in kb. Molecular markers on this 0.8 % TBE agarose gel are pBR322 *Eco*R/ *Eco*RI+*Pvu*II and *Alu*I/*Hin*fI respectively.

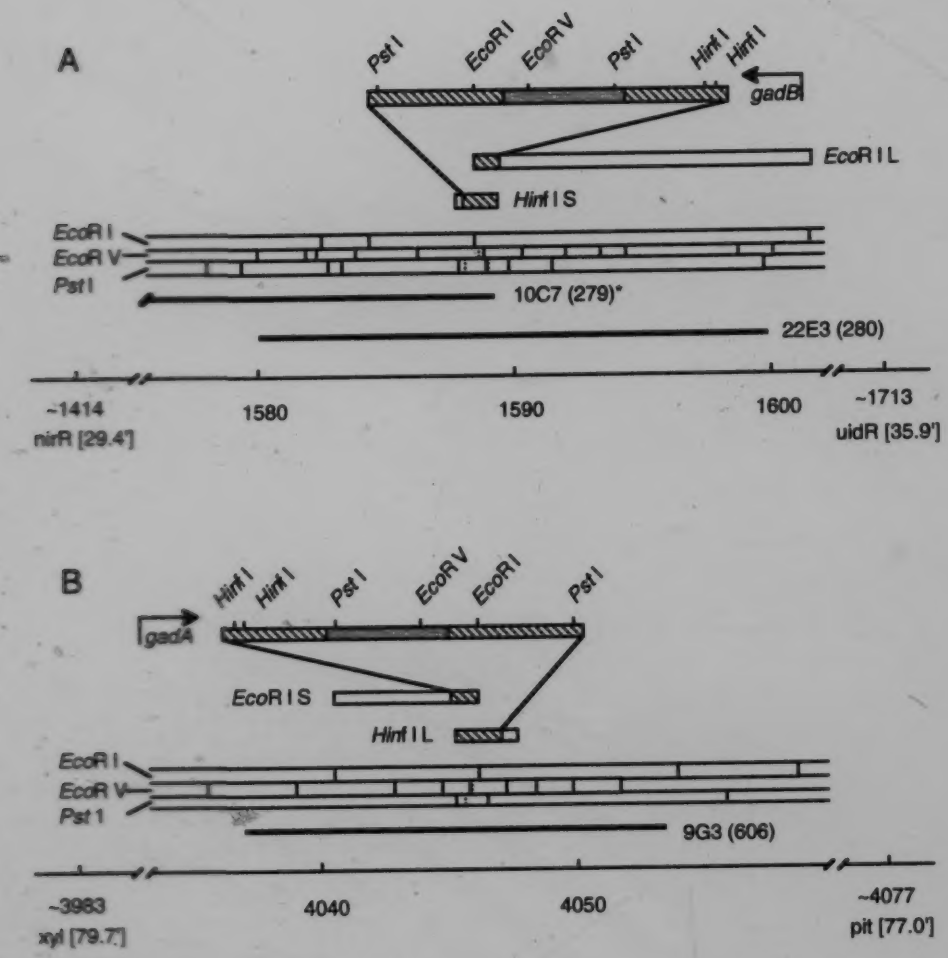


FIG. 2-7. Alignment of the GAD genomic clones with the *E. coli* physical map and corresponding miniset phage clones (Kohara, et al., 1987). Each pair of genomic clones (*Eco*RI L, *Hin*fI S or *Eco*R I S, *Hin*fI L) is shown in the center of A or B, and above each pair and connected by diagonal lines is an enlarged map of each *gad* ORF, with a number of restriction sites indicted. The arrow shows the direction of transcription. Hatched regions represent *gad* ORFs, stippled regions overlaid on the ORFs indicate the region encompassed by the original PCR probe, and the dotted lines within the re-drawn physical map shows the actual restriction sites seen in these genes. * indicates that the 10C7(279) phage clone has less overlap with 22E3(280) than is indicated in the original physical map figure (see Results).

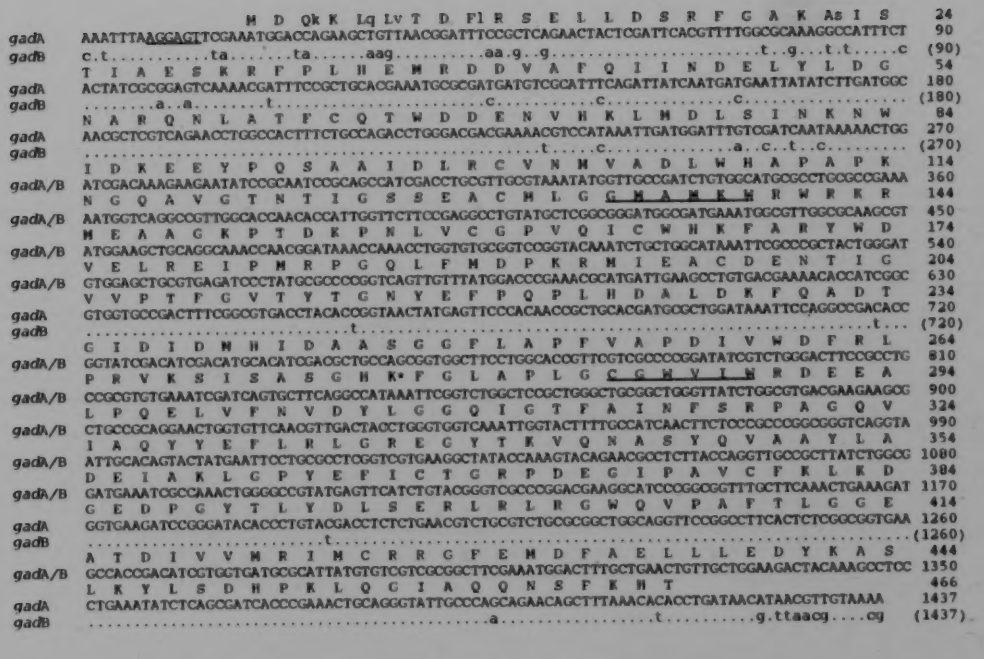


FIG. 2-8. Nucleotide and deduced amino acid sequences of *gadA* and gadB. All nucleotides and amino acids which are unique to *gadB* are shown in lowercase; otherwise, they are identical with those found in *gadA*. Regions of complete nucleotide identity are indicated by *gadA/B* at the start of the line. The putative Shine-Dalgarno sequences and the regions of protein sequence corresponding to the degerate primers used in the PCR are underlined. * indicates the lysine residue which binds pyridoxal phosphate.

Table 2-1. Comparison of amino acids at substituted positions for GAD α, GAD ß and GAD* (Maras et al., 1992).*a*

position	GAD α DNA-encoded sequence	GAD* protein sequence	GAD* DNA-encoded sequence	GAD ß DNA encoded sequence
3	Q	Q	NA*b*	K
5	L	L	NA	Q
6	L	L	NA	V
9	F	F	NA	L
22	A	A	NA	S
64	C	S	NA	C
73	H	R	NA	H
153	D	N	D	D
165	C	S	S	C
208	T	T	N	T
295	L	L	V	L
355	D	N	D	D

a Ambiguities exist between the protein and partial DNA-encoded sequences for GAD*, so both have been listed. The partial DNA sequences of GAD* spans the region from amino acids 148 to 431 of the 466-residue protein. Residues which are unique to GAD ß are listed.

b NA, not available.

Table 2-2. Sizes of *Eco*RI, *Hin*fI and *Pst*I restriction fragments from various strains of *E. coli* which hybridize to a *gadA* probe[a]

Strain (reference)	Restriction fragment(s) (kb)		
	*Eco*RI	*Hin*fI	*Pst*I
K-12			
J53-1 (Bachmann, 1972)	13.1, 5.6	2.2, 1.65	**1.02**
Wild type (Bachmann, 1972)	12.3, 5.5	2.2, 1.64	**0.96**
MG1655 (Guyer et al., 1981)	13.2, 5.6	2.2, 1.65	**0.97**
25404 (ATCC)	15.5, 5.6	2.2, 1.66	**0.98**
ECOR[b] (Ochman and Selander, 1984)			
20	13.0, 5.5	2.1, 1.60	ND[c]
26	16.5, 5.6	2.1, 1.54	ND
29	16.5, 5.6	2.2, 1.65	ND
37	6.3, 5.6	**2.2**	ND
46	14.0, 5.7	2.2, 1.64	ND
Other			
W (Davis and Mingioli, 1950)	16.5, 5.9	2.2, 1.68	**1.02**
11246 (Najjar and Fisher, 1952)	15.5, 6.0	2.2, 1.67	1.20, 1.10

a The probe is described in the legend to Fig. 2-6. Double-intensity bands are shown in boldface. ATCC, American Type Culture Collection.

b ECOR strain hosts: 20, steer (Bali); 26, human infant (United States); 29, kangaroo rat (U. S.); 37, marmoset (U.S. zoo); 46, Celebese ape (U. S. zoo).

c ND, not determined.

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Chapter 3

Immunization Of NOD Mice With Purified Native *E. coli* GAD Delays The Onset Of Spontaneous Diabetes, But The Same Result Is Also Obtained With A Control Antigen

Introduction

As noted in Chapter 1, injection of young NOD mice or BB rats with a variety of microbial products, including whole live BCG or heat-killed *M. tuberculosis* (i.e. CFA) can delay or even prevent the onset of spontaneous autoimmune diabetes. Several potential mechanisms can be proposed to account for this phenomenon, which broadly speaking might be antigen-specific or antigen non-specific. At the time that this work was begun, the human diabetes associated 64 kD autoantigen had recently been identified as the enzyme GAD (Baekkeskov et al., 1990). The fact that "immunization" with recombinant human or mouse GAD could prevent diabetes in NOD mice (Kaufman et al., 1993; Tisch et al., 1993; Elliott et al., 1994), together with our realization that certain bacteria (i.e. *M. tuberculosis* (GenBank z95389), BCG (Yasumizu et al., 1987) and *E. coli* (Strausbauch and Fischer, 1970)) also express a form of the GAD enzyme suggested the possibility that immunization with GAD^+ bacteria or extracts thereof might be inducing a cross-reactive immune response against islet expressed GAD, that is protective in nature (see Chapter 1). In fact, preliminary experiments related to this idea had already been carried out by our colleague Dr. B. Singh, using NOD mice and a commercially available preparation of *E. coli* GAD (ecGAD; from Sigma). For his experiments Dr. Singh had purified the ecGAD by passage over a single gel exclusion column. When the excluded fraction (termed "F1") was administered in IFA to 4 week old female NOD mice, it was found to be highly effective at preventing spontaneous diabetes (B. Singh, unpublished). However, also of note is the

fact that when a retrospective Western blot was performed on the F1 material using antiserum from the F1 immunized mice, bands at 66 and 80 kD stained more intensely than did the ecGAD band at 52 kD. In addition, numerous fainter bands were observed below 52 kD (S. Bhatti and J. Elliott, unpublished results), suggesting that the F1 material contained a number of contaminating proteins. Since the two bands at 66 kD and 80 kD were seen as minor contaminants in the SDS PAGE gels of F1, yet these bands stained intensely on a Western blot, it suggests that these two contaminants were highly immunogenic.

To explore the issue of antigenic cross-reactivity between *E. coli* and mouse GAD further, we wished to compare the protein sequences. Since the entire ecGAD sequence was not available, we first proceeded to clone the bacterial gene, as described in Chapter 1. With the gene and the deduced protein sequence in hand, we were able to continue with the investigations described in the present Chapter. These involved: i) comparison of the protein sequences for possible cross-reactive epitopes, and ii) attempting to repeat Dr. Singh's experiment on a larger number of animals, now using a highly purified preparation of ecGAD, and including also a second control antigen which had also been purified from *E.coli*. The motivation in the first instance was to determine if there was something unique about the use of ecGAD in the protection experiments, since this would support the notion of an antigen-specific mechanism.

For the protection experiments we had a choice of two possible preparations of *E. coli* GAD: i) native, endogenous protein produced in a fully folded, soluble form from any one of a number of different strains of *E.coli*, or ii) "recombinant" protein produced also in *E.coli*, but by over-expressing the protein using our cloned ecGAD gene and a T7 expression system. In the latter system the recombinant protein was fused at its' N-terminus with a (His)6 affinity tag, which would facilitate purification (Elliott et al., 1994).

However, with the T7 system the recombinant protein was overexpressed to such an extent that it formed insoluble inclusion bodies, which could only be dissolved using a powerful denaturant such as guanidinium hydrochloride, and the protein required refolding in the presence of small quantities of SDS to maintain solubility (S. Bhatti and J. Elliott, unpublished). We therefore chose to use the purified native ecGAD protein because it would maintain all naturally occurring B cell epitopes, which could potentially be important for efficient antigen presentation by B cells. Additionally, purified but native ecGAD most closely resembled the GAD present in the F1 material which had already been shown to prevent IDDM in NOD mice. For a control antigen, we chose a soluble recombinant protein called "Stoffel" fragment (a 548 amino acid, N-terminally deleted and exonuclease deficient form of *Taq* DNA polymerase) which was expressed in *E. coli* and purified over the same FPLC columns as was the ecGAD. The choice of the Stoffel fragment was largely a matter of convenience and availability, although it was important that the protein was roughly the same size as *E. coli* GAD, and that it showed no significant homology to *E. coli* or to mouse GAD.

Materials and Methods

Protein sequence comparisons: Sequences to be analyzed were obtained by translating our existing *E. coli* DNA sequences, or by downloading cDNA sequences from GenBank and translating these using DNA Strider 1.2, prior to importing into a GCG format. Global alignments were performed using the Best Fit program in the GCG Wisconsin Package, Version 8.1, UNIX 1995. More detailed alignments of limited regions of the proteins were also done using the SeqSee program (D. Wishart and R. Boyko, University of Alberta), also running on UNIX 1995.

Purification of native E. coli GAD and Stoffel fragment: The starting material for the preparation of *E. coli* GAD was purchased from Sigma (catalogue no. G-3757, lot 101H6811). This crude protein extract (105 mg) was dissolved in 10 ml of buffer A (50 mM Tris-HCl, pH 8.0, 10% glycerol) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 2.8 µg/ml Trasylol (Bayer, Mississauga, Ontario), and 0.2 mM pyridoxal phosphate (PLP; Sigma). PLP is the co-factor for the GAD enzyme which forms a Schiff base with the active site lysine. Binding of PLP is thought to stabilize the folded protein and conveniently also imparts a yellow color which simplifies detection of GAD during purification. A two stage sequential purification was performed using a Pharmacia dual Pump-500 FPLC and GP-250 Gradient Programmer. Eluted protein was monitored at 280 nm using a Single Path UV-1 detector (Pharmacia, Uppsala, Sweden). The initial ion exchange column (HR10 Mono Q, Pharmacia) was pre-equilibrated with buffer A and 1/3 of the dissolved protein (35 mg in 3.3 ml) was applied. Elution was performed using a gradient of NaCl in buffer A, at a flow rate of 0.75 ml/min, and collecting 1.5 ml fractions. Based on preliminary experiments, the initial NaCl concentration could be raised immediately to 0.18 M, after which the salt concentration was increased manually in a step-wise fashion (0.02 M increments per step, up to a concentration of 0.50 M). Eluted peaks

were allowed to return to baseline before proceeding to each subsequent step. Aliquots of each fraction were examined by SDS-PAGE and Coomassie blue staining. The purest fractions, highly enriched for the 52 kD GAD proteins, were obtained from the 0.20 M, 0.22 M, and 0.24 M NaCl steps. The column was stripped with 1.0 M NaCl in buffer A, re-equilibrated with buffer A, and run twice more in the same manner. Fractions containing GAD from the three column runs were pooled and concentrated to a final volume of 0.6 ml using a Centriprep 30 (Amicon, Beverly, MA). This concentrated pool was applied to a Superose 12 size exclusion column (Pharmacia), equilibrated and run in buffer A/ 0.3 M NaCl at a flow rate of 0.2 ml/min and collecting 0.4 ml fractions. SDS-PAGE analysis and Coomassie blue staining was again used to identify the fractions containing the purified GAD protein, which were pooled, and using a centricon 30 exchanged with PBS and then adjusted to a concentration of 2 mg/ml. For immunization/protection studies mice received 37.5 µl of the protein solution emulsified with an equal volume of IFA via a single footpad injection. The same amount of purified Stoffel fragment protein emulsified in IFA was used to inject control animals. Purification of the Stoffel fragment protein has been described elsewhere (Vainshtein et al., 1996). In subsequent experiments the purified GAD protein was show to be enzymatically active using a [^{14}C]-glutamate conversion assay (D. Markland and J. Elliott unpublished).

Preparation of heat killed BCG and related reagents: Live BCG was obtained from D. Kunimoto (University of Alberta) and grown in closed roller bottles at 37°C in Dubois Broth (Difco, Detroit, MI) adjusted to pH 4.6 and 10 mM glutamate for maximal GAD expression. Bacteria were grown to saturation (approximately 21 days of culture), harvested by centrifugation, washed twice in PBS, autoclaved twice, and then lyophilized. The preparation was re-suspended in 50 % IFA /PBS at 2.0 mg freeze-dried bacteria/ml. For protection studies mice received 50µl of this suspension in a single foot-pad injection. IFA and CFA (*M. tuberculosis* H37 RA) were obtained from Difco.

NOD mice and diabetes monitoring: Animals for these studies were obtained from the University of Alberta NOD/Alt breeding colony, maintained under virus-antibody free (VAF) conditions. For spontaneous disease studies mice were transferred to a clean conventional room to reduce costs and for the sake of convenience of access. Screening for diabetes involved daily urinary glucose testing using Tes-Tape (Eli Lilly, Indianapolis, IN) which when positive was then confirmed by blood glucose measurement, with blood from tail snips analyzed using a Glucometer Elite (Miles Canada Inc., Etobicke, ON). Animals with blood glucose levels above 15 mM were considered diabetic.

Results And Discussion

Comparison of GAD protein sequences: Fig. 3-1 shows a best fit alignment of the *E. coli* GADα and mouse GAD67 protein sequences. We chose to focus on mGAD67 since it is the predominant isoform found in murine islets (Roep et al., 1990; Michelsen et al., 1991; Pankewycz et al., 1991). The overall identity between the two proteins is 18%, and there is 47% similarity. This level of identity and similarity is quite unremarkable and is typically observed with proteins that are functionally related but evolutionarily divergent (or even functionally unrelated; see Stoffel comparison below). There is however one small region of much higher similarity between the two GAD proteins (underlined); this likely represents a major catalytic domain of the enzyme, and reflects the similar functions of the two proteins. This region is proximal to the HK "motif" (position 275 in ecGAD, 403 in mGAD67) which is known to bind pyridoxal phosphate, the vitamin co-factor required for GAD activity. For interest we did a similar comparison between ecGADα and the other isoform of mouse GAD (GAD65), with similar results (Table 3-1). In contrast, the GAD67 proteins from two more closely related organisms (mouse and humans) are 96% identical and 98% similar, and even two organisms as distant as mouse and drosophila show a remarkable 56% identity and 77% similarity between their GAD proteins (Table 3-1). This does suggest that there has been strong evolutionary pressure to conserve the GAD protein, and speaks to a critical role for the protein in metabolism as well as for neural function at least in higher organisms. We also compared the *E. coli* and mouse GAD sequences with the Stoffel sequence, since the latter was to provide a control antigen in our protection/immunization studies. For these functionally unrelated proteins the identity remains at the same low value of 18%, with similarity on the order of 45%.

Because we were especially interested in any potential shared T cell epitopes (i.e. linear peptide epitopes) between the *E. coli* and mouse GAD proteins, we did a second type of

comparison, where every possible 20 residue segment arising from the one protein was compared with every possible 20 residue segment from the other. The two best "fits" are shown in Fig. 3-2. Interestingly, the major similar peptide discovered in this analysis arises from the putative active site region, as already identified in and discussed for Fig. 3-1. The two regions have 7 of 14 consecutive residues showing identity, and 11 of 14 showing identify or similarity. The identity is even higher when the analogous region of mGAD65 is compared with the *E. coli* sequence (9 of 14 consecutive residues), suggesting that if it does represent a true cross-reactive epitope, it would be present in both of the isoforms of mouse GAD. This comparison yielded a second region of similarity, with 5 of 5 consecutive residues showing identity. Finding this level of identity is not particularly unusual when a search algorithm such as FastA is used to scan the protein sequence data base with any given protein sequence, but the core of identity could never-the-less represent an immunologically important epitope. When a similar comparison was done between *E. coli* GAD and the Stoffel fragment sequence, no region of comparable identity was found.

Purification of E. coli GAD: Fig. 3-3 shows SDS-PAGE analysis of the original GAD preparation purchased from Sigma, and compares it to fractions obtained after ion exchange chromatography, and after further purification by gel exclusion chromatography. From the original 105 mg of starting material, approximately 7 mg of purified ecGAD was obtained.

As a test of purity, the FPLC purified ecGAD protein was run on SDS-PAGE, Western blotted, and probed using antiserum I raised in rats immunized with the same protein preparation emulsified in R700 RIBI adjuvant (RIBI Immunochem Research Inc., Hamilton, MT). In this case we saw, in addition to a very strong 52 kD GAD band, only two additional faint bands at approximately 78 kD and 80 kD (D. Markland, unpublished

results). This contrasts with the results for the Western blot made with the "F1 fraction" as described in the introduction to this chapter.

The NOD/Alt colony and immunization with BCG in IFA: Since our laboratory had not done any previous studies on spontaneous diabetes in NOD mice, and since we had taken over the NOD/Alt colony upon Dr. Singh's departure from the University of Alberta, we felt that it was important to re-establish baseline incidence data for these mice before we undertook any large-scale protection experiments. Results for these studies are shown in Fig. 3-4 and Table 3-2. The incidence in the female mice was very high, with 93% of the animals becoming diabetic by about 150 days, which is similar to the highest incidence figures which have been reported world-wide. Also in keeping with the findings of other groups, the incidence in the males was much lower, at about 50% by 180 days.

With this preliminary assessment of the colony we did take the opportunity to carry out one small protection study as well, using heat killed BCG in IFA, and for interest and for sake of comparison with our subsequent protection studies this data is also included in Fig. 3-4. Essentially we found that when mice were injected with heat killed bacteria in IFA, both females and males were protected from diabetes (only 1 of 8 males and 4 of 17 females developed diabetes by about 225 days; in fact only the females reached statistical significance compared to the saline treated control group, Table 3-2). It is interesting to note that two additional BCG injected females turned diabetic after 400 days, suggesting that the "protective" effect of BCG may not necessarily be permanent, if one continues to monitor beyond 1 year of age, which is seldom done in published studies.

Motivation for this BCG protection experiment came from results we had obtained in collaboration with Dr. Rajotte's group (SMRI, University of Alberta), using the surgical model of transplanting healthy islets from young NOD mice into older diabetic animals

(what we and others have called the "disease recurrence" model; see Chapter 4). With this model, because of well established anti-islet autoimmune responses, the diabetic animals rapidly reject the syngeneic islet graft and hyperglycemia recurs typically within 7 to 21 days. However, this "autoimmune" rejection response can be delayed or even prevented if the recipient animals are at the time of surgery given an injection of CFA, or alternately a relatively massive dose of live BCG. Since BCG is an attenuated form of *M. bovis* closely related to *M. tuberculosis*, we were curious to know if heat-killed BCG in IFA (i.e. a "BCG based" version of CFA) would also protect in the disease recurrence model, but using a more reasonable dose of bacteria. Surprisingly our BCG based version of CFA did not significantly protect the recipient animals from recurrence of diabetes beyond what was observed with IFA control group (A. Sydie, M.Sc. thesis). The present experiment was undertaken to test the same BCG/IFA preparation for its' capacity to protect animals from developing spontaneous autoimmune diabetes. Indeed our data show that the BCG based version of CFA could provide stable long term protection from spontaneous diabetes. Taken together these results suggest that there are significant differences between the spontaneous and disease recurrence models, and/or that there are significant differences between heat killed *M. tuberculosis* and heat killed BCG in terms of immunostimulatory potency.

Immunization with ecGAD, Stoffel fragment, or CFA: The data from this series of experiments is shown in Table 3-3 and summarized in Fig. 3-5. The conclusion from this work is that early immunization with either ecGAD or CFA significantly delayed spontaneous disease onset relative to the saline control group, but a virtually identical result was also obtained by immunizing with an unrelated control protein, in this case the Stoffel fragment.

In contrast to the baseline incidence study in Fig. 3-4, the saline control group in this second experiment developed disease with somewhat slower kinetics (75% by about 175 days; 90% by 350 days). The incidence of disease in the various immunized groups was also somewhat lower than that found in the BCG "protected" animals from the previous experiment. This suggests that the kinetics of onset of spontaneous diabetes may vary somewhat over time, even in a single closed colony of animals.

Since the first is that the overall sequence identity between the *E. coli* and mouse GAD proteins is so low it suggests that the two proteins would not likely be immunologically cross-reactive. None the less, it was important for our hypothesis concerning antigenic cross-reactivity between microbial and mouse GAD that the formal sequence comparisons be completed. A caveat here is that with our increasing awareness of the plasticity of TCR/MHC/peptide interactions it is becoming clear that predictions of cross reactive T cell epitopes based simply on primary sequence alignments can be misleading. Several groups have identified cross reactive epitopes that would not have been predicted based on their primary protein sequences (Bhardwaj et al., 1993; Evavold et al., 1995; Kersh and Allen, 1996). Therefore the ultimate question of cross reactivity is best addressed functionally. However, given that a control antigen with low sequence identity (18 %) to both ecGAD and mGAD was able to prevent spontaneous disease, it suggests that mechanisms other than antigen cross reactivity may be responsible for the disease prevention observed with ecGAD. This interpretation is consistent with Ag recall proliferation studies with NOD spleen and lymph node cells, which failed to establish an immunological link between ecGAD and mGAD.

Using 20 aa peptides based on ecGAD from the regions of highest similarity between the *E. coli* and Mouse GADs (e.g. Fig. 3-1 and 3-2), no significant proliferation was observed with spleen or lymph node cells obtained from NOD mice primed with mGAD

whereas, a strong Ag recall response was observed relative to the priming Ag (J. F. Elliott and B. Singh, unpublished results). Similarly, in a pilot study I conducted which examined unprimed NOD spleen cell responses to antigens using methods previously published by another group (Tisch et al., 1993), I did not observe proliferation to recombinant ecGAD above levels seen with an irrelevant control antigen. Whereas, small but statistically significant increases in proliferation were seen relative to the same control antigen when contrasted to the results obtained with recombinant mGAD or islet lysates. Our interpretation of these data, is that there is no significant antigenic cross-reactivity between islets or mGAD and ecGAD

The second major goal of this chapter was to attempt to reproduce Dr. Singh's original experiment which demonstrated that immunization of young female NOD mice with *E. coli* GAD was highly effective at preventing the onset of spontaneous autoimmune diabetes. Recognizing that the original "F1" ecGAD preparation relatively crude, we went to some lengths to obtain as pure a protein preparation as possible. Also, given the observations previously reported with none-specific immunostimulation and disease prevention in NOD mice (Rabinovitch and Singh, 1995), in addition to a saline control group a second "negative" control group which was immunized with an irrelevant antigen (Stoffel fragment) which had also been purified from *E. coli* by methods similar to that used for ecGAD (Vainshtein et al., 1996). In view of the lack of any evidence in favor of an antigen cross reactivity between ecGAD and mGAD (or islets lysates for that matter) the disease prevention observed with ecGAD, Stoffel and CFA was more likely due to non-specific immunostimulatory effects. In the case of ecGAD and Stoffel this could be due to either the purified proteins, contaminants associated with the proteins or a combination of two possibilities in the context of IFA. IFA alone is known to provide some protection against diabetes but long term protection is generally only seen with combinations of IFA and antigen (Qin et al., 1993). One very real possibility is that a minor contaminating substance

common to both *E. coli*-derived protein preparations might be responsible for the protective effect. If such a substance were a ubiquitous, known immunostimulatory substance such as endotoxin (present in most proteins expressed in *E. coli*) it would still support the notion of an antigen non-specific mechanism for the protective effect. If on the other hand the factor were a protein such as heat-shock protein, the effect might still be antigen specific. These ideas underscore the fact that immunological studies which make use of "purified" proteins are limited by the purity of the antigen preparations, and they provide clear motivation for moving to synthetic peptides at the earliest possible opportunity. A more detailed discussion of the mechanisms involved with the non-Ag specific immune suppression is presented in the final Chapter 5. Given these conclusions and an understanding of the role of cytokines in both the protection from type 1 diabetes mediated through non-specific stimulation, as well as the role of cytokines in disease progression, I next looked at the direct role of these molecules in the modulation of type 1 diabetes.

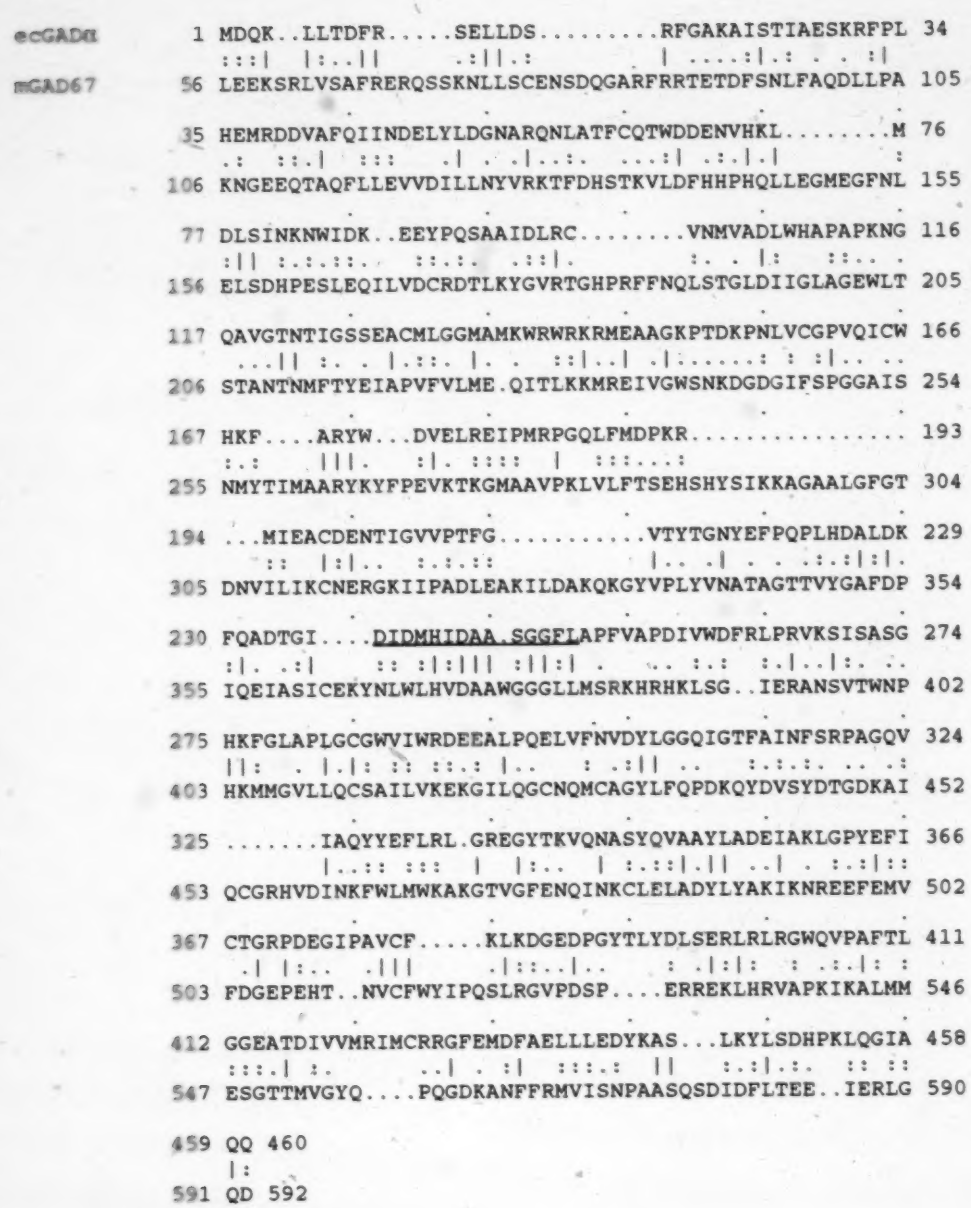

FIG. 3-1. Best Fit alignment of ecGADα versus mGAD67 proteins. Amino acid identities are indicated by a vertical bar with the degree of conservation of a substitution indicated by dots, where two dots represents highly conserved substitutions. The numbers identify the position of each amino acid. This alignment indicated a 18% identity and 47% similarity between these two proteins. The region of highest similarity is underlined.

TABLE 3-1. Summary of sequence identity and similarity[1] for selected GAD and control proteins[2].

Category	Protein Sequence Pairs	% Identity	% Similar	Source Figs.
GAD vs GAD	ecGADα[3] vs mGAD67	18	47	3-1
	ecGADα vs mGAD65	19	46	A-2-1
	mGAD67 vs hGAD67	96	98	A-2-2
	mGAD67 vs dmGAD	56	77	A-2-3
GAD vs Control	ecGADα vs Stoffel	18	46	A-2-4
	mGAD67 vs Stoffel	18	42	A-2-5

[1] Alignments were performed using the Best Fit program in the GCG, UNIX Wisconsin package, an example of which is shown in full in Fig. 3-1., and the remainder in the Appendix A-2.

[2] Abbreviations; ec, *E. coli*; m, mouse; h, human; dm, *Drosophila melanogaster*. Stoffel is a truncated *Taq* DNA polymerase.

[3] ecGADα and ecGADβ differ by only 5 amino acids, all lying in regions which are not conserved between the *E. coli* and mouse GAD proteins, hence only results with ecGADα are presented.

E. coli GAD versus mouse GAD

```
ecGADα      237  DIDMHIDAASGGFLAPFVAPD 257
                 :: :|:|||.|| |      .
mGAD67      366  NLWLHVDAAWGGGLLMSRKHR 386

ecGADα      237  DIDMHIDAASGGFLAPFVAPD 257
                 .| ||:|||.|| |      ..
mGAD65      358  KIWMHVDAAWGGGLLMSRKHK 378

ecGADα      443  ASLKYLSDHPKLQGIAQQNSF 463
                 .:   |||||.  :     ::
mGAD67      152  GFNLELSDHPESLEQILVDCR 172
```

FIG. 3-2. Regions of highest similarity between *E. coli* GAD and mouse GAD. Alignment notation is as described in Fig. 3.1. The core region of highest similarity is underlined.

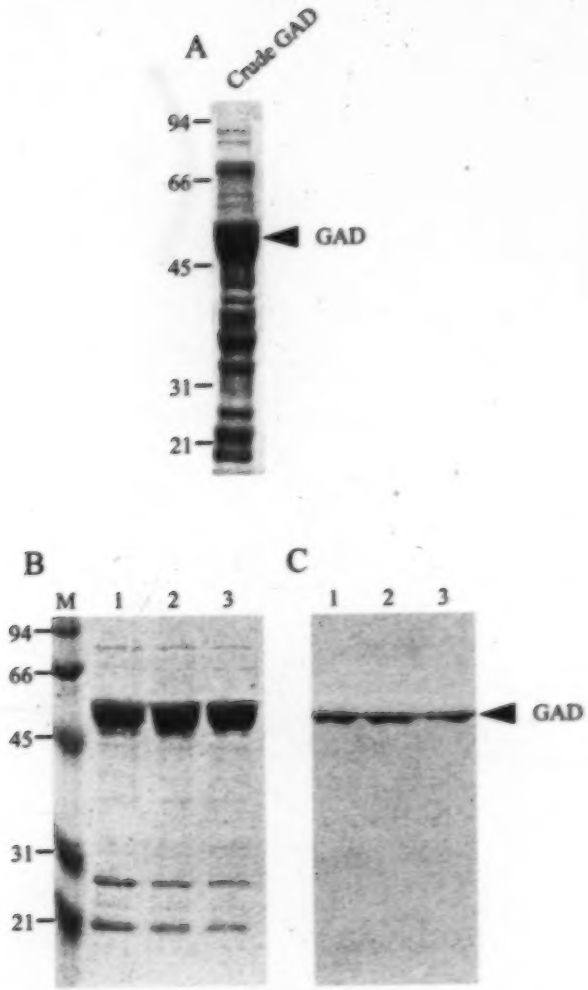

FIG. 3-3. Coomassie blue stained SDS PAGE analysis of the two step FPLC purification of *E. coli* GAD. Panel A, represents the crude bacterial extract obtained from SIGMA, dissolved in Buffer A prior to purification. The remaining two panels show representative fractions collected following sequential FPLC first with a HR10 Mono Q colum (B) and then with a Superose 12 column (C). Size demarcations for the gels are shown in kD and the gels in panels B and C have been scaled such that the marker in panel B serves both gels. Lane markers are as follows; M indicates protein size markers, B 1-3 represent fractions 58-60 and C 1-3 represent fractions III-2, III-3 and IV-1 which are described in the Methods and Materials section of this chapter.

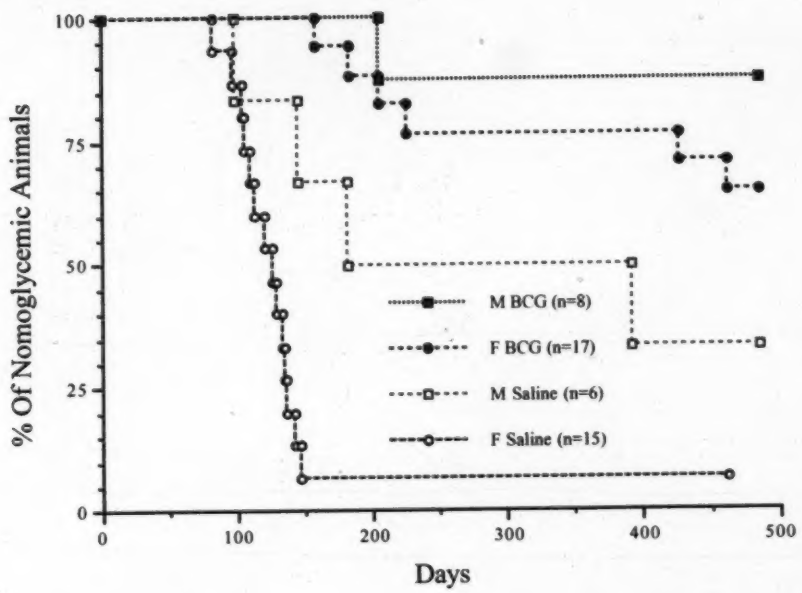

FIG. 3-4. Prevention of spontaneous diabetes with heat killed BCG in female and male NOD mice. Mice received a single 50 µl footpad injection of either physiological saline or BCG (100 µg) in 50 % IFA/saline at 6 weeks of age.

TABLE 3-2. Incidence of spontaneous diabetes in female and male NOD/Alt mice administered either saline or heat killed BCG in IFA[1].

Group		Days Nomoglycemic	n	Mean ± SEM	Median	*P* value[2] vs Saline
Female	Saline	82, 97, 105, 106, 111, 114, 120, 127, 129, 134, 135, 137, 142, 147, >462	15	143 ± 23	127	-
	BCG	159, 183, 206, 226, 427, 462, >462, >468x2, >471, >483x4, >484x2, >486	17	407 ± 30	468	<0.0001
Male	Saline	98, 145, 182, 392, >486x2	6	298 ± 72	287	-
	BCG	205, >483x7	8	448 ± 34	483	ns

[1] At 6 weeks of age mice received a single 50 µl footpad injection of either sterile physiological saline or autoclaved lyophilized BCG in 50 % IFA / saline, equivalent to 100 µg (dry weight) of bacteria.

[2] Student's *t* test, unpaired two-tailed; ns, not significant.

TABLE 3-3. Incidence of spontaneous diabetes in female NOD/Alt mice administered either saline, purified *E. coli* GAD in IFA, or purified Stoffel fragment in IFA[1].

Group	Days Nomoglycemic	n	Mean ± SEM	Median	*P* value[2] vs Saline[3]
Saline	68, 74, 92, 113, 138, 170, 175, 288, 352, >490	10	196 ± 44	154	-
ecGAD	175, 242, >479x2, >482x2, >490x2, >491, >507x2	11	439 ± 35	482	<0.001
Stoffel	175, >479x2, >482, >490x2, >491x2, >507, >509	10	459 ± 32	490	<0.0001
CFA	320, 472, >479, >482, >490, >491x2 >507x2, >509	10	475 ± 18	491	<0.0001

[1] At 4 weeks of age mice received a single 75 µl footpad injection of either physiological saline, CFA (75 µg *M. tuberculosis* H37Ra), FPLC purified ecGAD protein (75 µg) in 50 % IFA / saline emulsion, or FPLC purified Stoffel protein (75 µg) in 50 % IFA / saline emulsion

[2] Statistical difference between the various groups was determined by one-factor ANOVA.

[3] There was no statistically significant difference between the ecGAD, Stoffel, and CFA immunized groups.

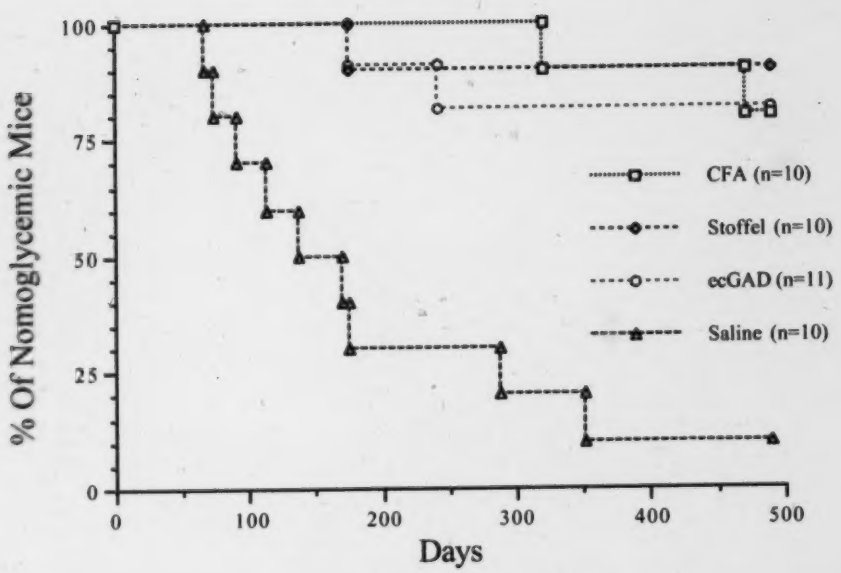

FIG. 3-5. Prevention of spontaneous diabetes with *E. coli* GAD and other microbial antigens in female NOD mice. At 4 weeks of age the mice received a single 75 µl footpad injection of either ecGAD or Stoffel protein (75 µg) in 50 % IFA/saline emulsion, CFA (75 µg *M. tuberculosis* H37Ra) or physiological saline.

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Chapter 4

IL-4 or IL-10 Expressed From Adenovirus Transduced Syngeneic Islet Grafts Fails To Prevent ß Cell Destruction In Diabetic NOD Mice

A version of this chapter has been published.

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Introduction

Pancreatic islet replacement, as a therapy for lost ß cell function due to Insulin Dependent Diabetes Mellitus (IDDM) must overcome the autoimmune destruction of pancreatic ß cells (Castano and Eisenbarth, 1990) in addition to the allo or xeno rejection encountered in transplantation. There is evidence of recurrent autoimmunity with human allogeneic islet transplantation in spite of immunosuppressive therapy (Stegall et al., 1996) and long term islet function post transplant has generally been poor (Warnock and Rajotte, 1992). Therefore, strategies that will be effective in the down regulation of the autoimmune process as well as allo and xeno rejection will likely rely on manipulation of the immunological regulatory factors and their analogs.

Animals models such as the NOD mouse display many similarities to the human disease (Kikutani and Makino, 1992) and have permitted new insights into the immuno-pathology of autoimmune diabetes. Through the use of these models it has become clear that T cells play a central role in disease development as demonstrated by spleen cell transfer (Wicker et al., 1986; Christianson et al., 1993) and antibody depletion studies (Harada and Makino, 1986; Koike et al., 1987). In turn T cells are more frequently identified as belonging to functional subsets identified by the patterns of cytokines they produce (Mosmann and Sad, 1996). The inter-regulation between T cells and identification of specific counter balanced

CD4 Th subsets, such as Th_1 versus Th_2 (Mosmann and Moore, 1991) and more recently the analogous CD8 subsets (Mosmann and Sad, 1996), has focused immunological thought on a few key regulatory cytokines within this paradigm. IFN-γ, IL-12 or TGFß in most experimental systems favors the development of cell mediated Th_1 like responses, whereas IL-4 favours a humoral Th_2 like response (Seder and Paul, 1994). IL-10 is thought of as another key cytokine that is produced by Th_2 clones, but, unlike IL-4, IL-10 inhibits cytokine synthesis from Th_1 cells in vitro (Fiorentino et al., 1989) rather than influencing naive T cell differentiation.

As reviewed by Rabinovitch (Rabinovitch, 1993; Rabinovitch, 1998), there is considerable support for the notion that in NOD mice IFN-γ is associated with ß cell destruction and that protection from disease is linked to the Th_2 products IL-4 and IL-10. However, as is outlined below it is increasing clear that the assignment of a cytokine to either a disease inducing or preventing category can be complex at least with IDDM. Also, since the number of T cell subsets continues to expand with further characterization of specific disease models and systems (Mosmann and Sad, 1996), a simple understanding of causal links between one defined subset and a particular outcome is difficult. None the less the Th_1/Th_2 paradigm has at least structured the debate and provided some testable hypotheses.

The observations that link specific cytokines and T cell subsets to diabetes pathogenesis include the following. Diabetes in NOD mice is prevented with the administration of anti-IFN-γ antibody (Debray-Sachs et al., 1991). A higher frequency of IL-4 producing cells is found in adjuvant protected NOD islet grafts versus an elevated frequency of IFN-γ producing cells in unprotected transplants (Shehadeh et al., 1993; Suarez-Pinzon et al., 1996). Exogenous IL-12, which stimulates IFN-γ production (Trinchieri et al., 1992), will speed the development of diabetes in NOD mice (Trembleau et al., 1995). Prevention

of spontaneous disease is evident during systemic administration of either IL-4 (Rapoport et al., 1993) or IL-10 (Pennline et al., 1994). Th_1 like islet specific NOD T cell clones (Haskins and McDuffie, 1990; Peterson and Haskins, 1996) and lines (Katz et al., 1995) induce disease when administered to NOD mice; whereas Th_2 cell lines with an identical T cell receptor (TCR) prevent disease (Katz et al., 1995). However, these Th_2 cell lines are apparently unable to prevent diabetes in the presence of their Th_1 partners (Katz et al., 1995), and Th_1 islet specific clones with variant TCRs differ in their ability to induce disease in the absence of CD8 positive T cells (Peterson and Haskins, 1996). The Th_2 cytokine picture is further complicated by the fact that IL-10, when expressed as a transgene from the pancreatic ß cells (Ins-IL-10) (Wogensen et al., 1994) or α cells (Moritani et al., 1994), accelerates disease in NOD mice. Even in the absence of any other diabetes susceptibility alleles, Ins-IL-10 B10 mice homozygous for the NOD MHC (H2g7) develop insulitis and diabetes (Lee et al., 1996).

More in line with the exogenous cytokine experiments (Rapoport et al., 1993), are the most recent transgene experiments with pancreatic expression of IL-4 in a NOD background (Mueller et al., 1996). These IL-4 transgenic mice have reduced insulitis, no diabetes, are resistant to disease induction by diabetic spleen cells, but islet grafts expressing IL-4 offer no protection against disease recurrence when transplanted into diabetic syngeneic recipients. Clearly, the complex interactions of these cytokines in vivo goes beyond our understanding of the in vitro properties of these molecules.

We selected adenoviral vectors for our cytokine experiments because these constructs represent a well characterized, highly efficient gene transfer technology (Becker et al., 1994), with demonstrated expression in many non dividing primary cell types including pancreatic rat (Becker et al., 1993), mouse (Csete et al., 1995), and human islets (Csete et al., 1994). The E1 deleted vectors can be engineered to express genes ≤ 3 kb and grown to

a high titer. Compared with transgenic technology, it is relatively easy to alter the expression level of the selected gene product with adenoviral vectors simply by varying the virus to target cell ratio. The most serious drawback with adenoviral constructs for many gene transfer applications is their immunogenic potential (Engelhardt et al., 1994). However, it has recently been demonstrated in the rat that it is possible to generate a state of "unresponsiveness" to an adenoviral construct by previous intrathymic injection of the vector (DeMatteo et al., 1995). In spite of the previous work with adenoviruses and islets, much needs to be worked out in terms of optimizing protocols for islet infection and for determining the duration of exogenous gene expression in vivo. The central questions addressed in this work are the following: How efficacious are adenoviral expression vectors for gene transfer into mouse pancreatic islet grafts, and can adenoviral expression of either IL-4 or IL-10 from a syngeneic graft prevent disease recurrence in diabetic NOD mice?

Materials and Methods

Media and General Reagents: All media and fetal bovine serum (FBS), with the exception of the serum used during the viral plasmid cotransfections (FBS from Hyclone, Logan, UT), was purchased through GibcoBRL (Burlington, ON). COS 7 and 293 cells were cultured in DMEM high glucose media, 10 % FBS. Islets were generally cultured in Ham's F10 supplemented with 10 mM glucose, 50 µM isobutylmethylxanthine (IBMX; ICN Biomedicals, Inc., Costa Mesa, CA), 0.5% BSA (fraction V; Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 10 mM nicotinamide (BDH Biochemical, Poole, England), 100 U/ml penicillin and 100 µg/ml streptomycin. However, the glucose stimulated static islet insulin release assays were conducted in a custom formulation of glucose free RPMI 1640 that was supplemented to the desired glucose concentrations detailed below. Restriction endonucleases and buffers were supplied by either Boehringer Mannheim (Laval, Quebec) or New England Biolabs (Beverly, MA). Trypsin and DNase were purchased from the former whereas, T4 DNA ligase was obtained from the latter. All other reagents were supplied by BDH, Fisher Scientific (Ottawa, ON) or Sigma and were at least of analytical grade.

Animals: Five- to eight-week-old male Balb/c mice were purchased from the University of Alberta Health and Laboratory Animal Services and housed in Microisolators in a conventional facility at the University. Recipient Balb/c mice were rendered diabetic by intravenous injection of 90 mg/kg body wt alloxan (Sigma; freshly dissolved in 1 mM hydrochloric acid) 4-5 days before transplantation. All animals entering the study exhibited non-fasting blood glucose levels above 20 mM. Blood was obtained from the tail vein for the glucose assays (Glucometer Elite glucose meter, Miles Canada Inc., Etobicke, ON). NOD/LtJ mice were purchased from Taconic Farms (Germantown, NY). Recipient female NOD mice were maintained under virus-antibody free (VAF) conditions until diabetes

onset, at which time they were transferred to conventional housing as above. Screening for diabetic female NOD mice was initiated at 9 wk. Daily urine glucose was determined using Tes-Tape (Eli Lilly, Indianapolis, IN) which when positive was then confirmed with a blood glucose measurement. Animals with blood glucose levels above 15 mM were considered diabetic and were then maintained on Ultralente beef-pork insulin (Cannaught Laboratories, Toronto, ON) for at least 2 wk prior to transplant. Insulin was administered as required to maintain a minimal blood glucose range between 6 mM and 15 mM. All animal rooms were climatized and the mice had free access to water and pelleted food.

Viral Preparations: Viral constructs were E1 deleted, replication deficient, and adenovirus serotype 5 derived. CMVßGal encodes a nuclear localizing form of *E. coli* ß galactosidase under the control of a human cytomegalovirus (CMV) promoter enhancer (Bonnerot et al., 1987). The ßGal adeno construct (AdCMVßGal) was provided by C. Newgard (University of Texas, TX) and is described in Herz and Gerard (Herz and Gerard, 1993). Addl 70-3 (Bett et al., 1994) was used as a control construct which does not express a reporter gene and was provided by F.L. Graham (McMaster University, ON). General methods outlined in Becker *et al.* (Becker et al., 1994) and Graham and Prevec (Graham and Prevec, 1991) were used in engineering and plaque purification of recombinant adenovirus. AdCMVmIL-4 and AdCMVmIL-10 were produced by calcium phosphate cotransfection (Transfection MBS, Mammalian Transfection Kit, Stratagene, La Jolla, Ca.) of the shuttle plasmid pCA14 (Hitt et al., 1995) with the viral plasmid JM17 (McGrory et al., 1988) into 293 cells (Graham et al., 1977) followed by rescue of recombinant virus. Cloning of murine IL-4 (mIL-4) and IL-10 (mIL-10) into the shuttle plasmids is described below and followed standard molecular biological procedures (Ausubel, 1995).

Cytokine secretion was confirmed by ELISA of supernatants from COS 7 cells transduced with plaque purified recombinant adenovirus. Recombinant virus was

expanded in large scale plate cultures of 293 cells which were harvested once they demonstrated the appropriate degree of cytopathic effect. Cells were lysed with two rounds of freeze thawing in a minimal volume of DMEM high glucose media 10% FBS. Cellular debris was cleared by centrifugation at 4,000 *g* for 10 min at 4° C and the stock was brought to 10 % glycerol prior to freezing at -70°C. Viral titers were determined by plaque assay on 293 cells and were typically in the range of 10^9 to 10^{10} PFU/ml.

IL-4 and IL-10 Shuttle Plasmids: The IL-4 encoding shuttle plasmid was derived by sub-cloning the IL-4 open reading frame (ORF) from a Xba1/Eco R1 delimited PCR engineered expression cassette, into the corresponding sites of pCA14. Primers used in the PCR were 5' CTCTCTTCTAGACCACCATGGGTCTCAACCCCCAGC 3' and 5' CTCTCTGAATTCCCACCCTACGAGTAATCCATTTGCATG 3' with annealing, extension and denaturation temperatures at 50°, 72° and 94°C respectively, each for one min, in a reaction volume of 100 µl. Buffer, primer, nucleotide and *Taq* DNA polymerase concentrations were as recommended by Perkin-Elmer Cetus (Norwalk, CT). For the IL-10 sub cloning, pCA14 was modified by the insertion of the Xba1/Eco R1 fragment from the polylinker of pJFE14 (Elliott et al., 1990) into the corresponding sites of pCA14. Designated pCA14 Not1 (Fig. 4-1), this plasmid has several new features including a Not1 site, which permitted the cloning of a Xba1/Not1 delimited IL-10 expression cassette described in Li *et al.* (Li et al., 1994). The target plasmids used for the PCR reactions were obtained from T. Mosmann (University of Alberta, AB) and the PCR constructs were sequenced on one strand to confirm the ORFs.

Islet isolation, infection and dissociation: Islets were isolated using collagenase digestion and Ficoll purification as described in Wang *et al.* (Wang et al., 1992), by SMRI personnel in the Department of Surgery at the University of Alberta. Balb/c islets were obtained from 5-to 8-week-old males whereas NOD islets were restricted to 5-week-old male donors.

Freshly isolated islets were hand picked, aliquoted into groups generally of 400-500 islets, and centrifuged at approximately 180 *g* for 1 min and the medium was then removed with the exception of a thin film covering the islets. 0.5 µl/islet of transfection virus stock (prepared as above and thawed just prior to use) was used to transfer the islets to a single well of a 24 well plate. A second aliquot of 0.5 µl/islet of the infective dose was then used to rinse any remaining islets into the same well. Islets and virus were incubated for 1 h at 37° C, 5% CO_2, washed three times to remove free virus and either prepared for transplant or put into tissue culture. The same volume/islet was used during infections with smaller aliquots of islets. The concentration of the working viral stock was adjusted to vary viral load/islet and an appropriate incubation plate selected for smaller volumes. To determine the percentage of Balb/c islet cells infected with AdCMVßGal at 2.5 x10^3 PFU/islet and determine cell recovery, the islets were dissociated 48 h post transduction prior to staining for ß galactosidase. Single cell suspensions were produced by gentle agitation in calcium-free media containing trypsin (15 ug/ml) and DNase (4 µg/ml) (Korbutt and Pipeleers, 1992; Pipeleers et al., 1985; Pipeleers and Pipeleers-Marichal, 1981). A Burker chamber was used for cell counts (Korbutt and Pipeleers, 1992; Pipeleers et al., 1985).

Insulin Release Assays: The in vitro function of transduced islets was determined by a glucose stimulated static insulin release assay (Korbutt and Pipeleers, 1992). Forty-eight hours after infection, cultured islets were recovered, washed and duplicate aliquots of 50 islets were processed for time zero total cellular insulin determinations. Additional duplicates were placed in 1.5 ml of either 2.8 mM glucose or 20.0 mM Glucose RPMI 1640 supplemented with 2 mM L-glutamine, 0.5 % BSA and incubated for 120 min at 37° C. Time zero islet aliquots were stored in 2 mM acetic acid containing 0.25 % BSA and a cell free sample of culture supernatant was harvested following the incubation period. The acidified islet preparations were sonicated and centrifuged at 800 g for 15 min at 4° C. Centrifuge and tissue culture supernatants were stored at -20° C until assays were carried

out using a rat insulin RIA (Pharmacia, Uppsala, Sweden) that cross reacts with mouse insulin. The results are expressed as percent release, or the percent of the total cellular insulin content at time zero observed in the tissue culture supernatant. Stimulation indices were calculated by dividing the percent release at 20 mM glucose by that at 2.8 mM glucose.

With AdCMVmIL-4 infected NOD islets, an additional in vitro assay was performed in which two virus doses were used (25 PFU/islet and 2.5 $x10^3$ PFU/islet) with the lower of the two employed for the control virus, Addl 70-3. Immediately following transduction, aliquots of 50 islets were divided between pre-incubation DNA samples or islets intended for tissue culture. Cultured islets were incubated in Ham's F10 medium for 48 h at 37° C after which supernatants were collected for secreted insulin and IL-4 assays. The islets were harvested for post incubation cellular insulin and DNA content determinations. DNA assays for islet cell lysates were determined by methods published previously (Korbutt et al., 1996) using Pico Green, a florescent double strand sensitive DNA stain (Molecular Probes, Inc., Eugene, OR). The insulin content of the medium was again expressed as a percentage of the total content. However, total content here was defined as tissue plus medium.

Islet Staining For β Galactosidase: Dissociated islet cells or whole islets were washed in phosphate-buffered saline (PBS), fixed for 5 min at room temperature (RT) in 1 % formaldehyde, 0.2 % gluteraldehyde and washed three times with PBS prior to overnight (16 h) staining with X-gal (American BioOrganics, Niagara Falls, New York) at 37° C. The stain solution consisted of 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride, 0.3 mg/ml X-gal from a 2 mg/ml stock in dimethyl formamide (Aldrich, Milwaukee, WI) brought to volume with PBS. No

background staining was observed under these conditions, however this was not the case with other X-gal and Blue Gal stain formulations that were tried.

Islet Cytokine Expression and ELISA: Transduced and control islets were cultured in Ham's F-10 medium described above at 37° C. Larger cultures (e.g. 1000 islets in 12 ml media) were sampled daily by removing 0.5 ml of supernatant followed by a supplement of 0.6 ml of fresh media to compensate for sampling losses. Smaller cultures of 50 islets in 0.5 ml volumes were sampled once after a 48 h incubation. Supernatant samples were passed through a 0.45 μm syringe filter (Millipore Corp., Bedford MA) to remove islet cells and stored at -70° C until analyzed. Double monoclonal antibody (mAb) sandwich ELISAs were used to determine the concentration of cytokine secreted into tissue culture medium. First and second Abs for each cytokine were: IL-4, 11B11 and BVD6-24G2 (PharMingen, San Diego, CA) and IL-10, SXC1 and SXC2 (Mosmann et al., 1990). Secondary mAbs were biotinylated and a peroxidase-conjugated streptavidin complex (Jackson Immuno Research Laboratories Inc., West Grove, PA) provided the enzyme linkage for the assay. Primary mAb was dissolved in PBS while the secondary mAb and conjugate were dissolved in PBS, 1% BSA and 0.05 % Tween 20. Initially a 10 min 37° C blocking step with PBS and 1% BSA was included after coating the ELISA plate with primary mAb. The blocking step was later omitted when primary mAb and sample incubations were changed from 30 min at 37° C to 1 h at RT in order to reduce background. Similarly, the secondary mAb and conjugate incubations were changed from 30 min at 37° C to 30 min at RT. Plates were washed twice with PBS and 0.05 % Tween 20 (PBST) after each incubation. Azino-bis-ethylbenz-thiazoline-sulfonic acid (ABTS; Sigma) was typically used as a substrate for development of the reactions which were read at 405 and 490 nm. Tetramethyl-benzidine (TMB; Sigma) was used as a alternate substrate to increase sensitivity, primarily for control samples, and these reactions were read at 490 and 450 nm. An additional three washes with PBST were added between incubations

when TMB was used as a substrate. Cytokine levels were determined as ng/ml of supernatant which were then converted to ng/islet by compensating for the volume and number of islets in a particular assay.

Transplants And Monitoring: Three transplant studies were undertaken. In the first, control and transduced (2.5 x10^3 AdCMVßGal PFU/islet) Balb/c islets in groups of 400 were transplanted under the kidney capsule of Halothane-anesthetized alloxan induced diabetic syngeneic recipients. Non fasting blood glucose was determined 3 times a week initiated 48 h post transplantation. Nephrectomies were performed on both control and transduced islet grafts at several time points over the 8 week study to confirm graft dependence for glucose normalization and examine expression of ß galactosidase. For the NOD mouse AdCMVmIL-10 experiment, the control animals received a graft transduced with AdCMVßGal and both viruses were used at 2.5 x10^3 PFU/islet. Groups of 500 control or AdCMVmIL-10 infected NOD islets were transplanted under the kidney capsule of fully diabetic syngeneic female recipients. Blood glucose was monitored daily until disease recurrence. With the NOD mouse AdCMVmIL-4 study, two virus doses were used (25 PFU/islet and 2.5 x10^3 PFU/islet) with the lower of the two employed for the control virus, Addl 70-3. Fully diabetic NOD female recipients initially received a 400 islet syngeneic graft in each of its kidneys, for a total of 800 islets. One of each pair of islet grafts was non-transduced, while the other was infected with AdCMVmIL-4 at one of the two doses or the Addl 70-3 control virus. Blood glucose was monitored daily for the first week after transplant and three times a week subsequently. When animals exhibited blood glucose above 15 mM on two consecutive monitoring days they were considered diabetic and were again maintained on insulin until a second surgery could be scheduled. All animals from the three groups in the IL-4 study then received an additional non-transduced syngeneic 500 islet graft and were again monitored until disease recurrence.

Islet Graft Preparation and Immunohistochemistry: At the time of nephrectomy, kidney sections that contained engrafted islets were snap frozen in Tissue-Tek O.C.T. (Miles Canada Inc.) and stored at -70° C. Cryosections were cut at 6-8 µm, transferred to APTEX (Sigma) coated slides and briefly air dried. Slides were then immersed in PBS and 0.25 % Gluteraldehyde for 15 min at RT, washed three times in PBS for 1, 5 and 10 min respectively, then stained with X-gal solution overnight as above. Sections were then either lightly counter stained with eosin or processed for immunohistochemistry to detect insulin (Korbutt et al., 1996) and counter stained with Harris's hematoxylin.

Statistical Analysis: Data are expressed as means ± SD of *n* independent observations. Statistical significance of differences was determined with a two-tailed unpaired Student's *t* test at a minimum $P < 0.05$.

Results

Balb/c In vitro Studies: Counts performed on single cell suspensions of dissociated islets from each of the isolations demonstrated that cell recovery/islet was not significantly different between the control (973 ± 49) and AdCMVßGal infected islets (961 ± 48). These cell recovery numbers likely represent an under estimate for both groups since cells at the periphery of the islets are at greater risk of lysis and may be lost during the procedure. Rounding the estimates to 1,000 cells/islet, the multiplicity of infection (MOI) at 2.5 x10^3 PFU/islet was estimated to be 2.5. When islet cells were treated with the vital stain neutral red as an additional indicator of viability, both groups were in excess of 90 % positive 24 h post dissociation. Overnight staining of the islet suspensions with X-gal determined that 51 ± 4 % of the islet cells were positive for ß galactosidase, with no background stain evident in non-transduced cells (Fig. 4-2). It should be noted that the low variance observed in this series of transductions was not always the case. Variability in collagenase digestion times or temperatures may result in inconsistent transduction efficiencies. However another important parameter was the time in tissue culture at 37° C prior to infection. A drop in transduction efficiency on the order of 40 % was observed if islets were cultured overnight at 37° C prior to infection (data not shown). When whole islets transduced with AdCMVßGal were stained with X-gal after 48 hours in culture, a proportion of the islets in any preparation would fail to exhibit any blue stain. It was also evident that cells on the periphery of the islets were more likely to be transduced than those at the core of the islet (data not shown). In vitro viability of the islets following infection with recombinant adeno virus was further demonstrated when transduced and control islets showed no significant difference in basal or glucose stimulated insulin release (Fig. 4-3 A).

In Vivo Function of AdCMVßGal Transduced Islets: Alloxan induced diabetic Balb/c recipients were transplanted with either 400 transduced (n=15) or control (n=7) islets

under the kidney capsule. Diabetic mice exhibited blood glucose above 20.0 mM before transplant and generally achieved nomoglycemia (< 8.0 mM) within two days, which represented the first post-transplant blood glucose determination. Both transduced and control animals remained euglycemic over the 8 week study (Fig. 4-3 B). Animals nephrectomized at various time points ranging from 3 to 55 days, demonstrated graft dependence for glucose normalization. Positive staining for ß galactosidase was seen to decrease over time, however reporter gené product was still easily detectable at 8 wk (Fig. 4-4). An accumulation of mononuclear cells was evident adjacent to but not invading some of the transduced grafts (e.g. Fig. 4-4 A), but this did not apparently compromise the in vivo function of the grafts over the study period.

Cytokine in vitro studies: As with the previous static glucose response tests, no significant difference was seen between each of the pairs of transduced or control islets with either AdCMVmIL-4 or AdCMVmIL-10 (Fig. 4-5). Basal insulin release was higher with control and transduced NOD islets with either cytokine by a factor of 3 to 4 relative to the data shown in Figure 4-3 A for AdCMVßGal in the Balb/c islets. However higher basal values were also observed in a second set of AdCMVßGal static assays in Balb/c islets. This indicates that the elevated basal insulin releases were not a function of the NOD islets but were rather due to a differences in the isolations and therefore the general state of the islets, or the tissue culture conditions. For example, the higher basal insulin secretion was compensated for in the case of the IL-4 statics; with an elevated insulin release at 20.0 mM (Fig. 4-5 A), but not in the IL-10 statics (Fig. 4-5 B) involving a different isolation. Hence, stimulation indices in the AdCMVmIL-4 experiments for transduced and control NOD islets were comparable to those seen in the AdCMVßGal Balb/c series (Fig. 4-3 A) but lower by a factor of 3 during the comparable AdCMVmIL-10 experiments with NOD islets. The important observation, however, is that control and virally transduced islets behave similarly for any given islet preparation.

An additional set of insulin release assays were performed for AdCMVmIL-4 transduced NOD islets. A comparison over time in terms of insulin release and islet recovery, as determined by DNA content, was made between three experimental groups in tissue culture over a 48 h period. Islets were transduced with AdCMVmIL-4 at either 25 PFU/islet or 2.5 x10^3 PFU/islet or with a control virus (Addl 70-3) at the lower dose. As with the glucose stimulated assay there was no significant difference between the three groups in terms of the parameters measured.

Non-transduced Balb/c and NOD islets did not secrete detectable levels of IL-4 or IL-10 and cytokine secretion in transduced islet cultures was found to be variable between isolations. None the less, adenoviral expressed recombinant cytokine production could still be shown to be a viral dose dependent phenomenon (i.e. PFU/islet), with accumulation of cytokine in tissue culture media over time (Fig. 4-6). NOD islets had a tendency to express less cytokine than Balb/c islets but variability in the data made evaluation of this difficult (Table 4-1).

AdCMVmIL-4 Transplants: A preliminary study involving diabetic NOD mice receiving a single syngeneic AdCMVmIL-4 transduced islet graft under the kidney capsule suggested that this adeno construct might actually accelerate graft destruction. Although a number of technical concerns with respect to this set of transplants complicated the interpretation of this initial result, we decided to adopt an alternate experimental design which could reveal protective effects of IL-4 that might not be apparent using a single graft protocol.

In experiments in which cytokines were used to enhance the immunogenicity of tumors in mice, it was reported that IL-4 had an in vivo eosinophil recruitment property (Tepper et al., 1992). This could potentially result in the non-T cell dependent destruction of islets

expressing IL-4 even if this Th_2 cytokine were able to down regulate the autoimmune process. As a result of this consideration, a double transplant model was utilized in which mice received a syngeneic islet AdCMVmIL-4 transduced graft under one kidney capsule while simultaneously receiving a second non-transduced graft of equal mass in the opposite kidney. In the event that transduction with AdCMVmIL-4 resulted in protection of the graft, histological examination would reveal the extent to which the altered response could be generalized to the non-transduced graft. The experimental design could also reveal the potential of IL-4 to affect Th lineage development toward the Th_2 pathway over time, even if the graft expressing the recombinant cytokine were impaired in a manner unrelated to the autoimmune process. This assumes that any protective regulatory events that might result from expression of IL-4 in the presence of islet antigen at the site of the graft would expand to influence events at the non-transduced graft. To allow for the possibility that ß cell destruction in the non-transduced graft was more rapid than the induction of protection from the IL-4 transduced graft site, a second non-transduced graft would be provided near the original site of the transduced graft in the event of disease recurrence following the first pair of grafts. In addition to a control virus, two viral doses of AdCMVmIL-4 were also incorporated into the study to examine the role that local concentration of cytokine might have.

With each isolation aliquots of 50 islets were used to monitor cytokine expression in vitro which was evident with each series of transductions. All animals demonstrated normal blood glucose generally within 24 h, but the kinetics of disease recurrence was not significantly different between the three groups (Fig. 4-7 A). In contrast to the preliminary work using a single graft, these results with the double islet transplant protocol made it clear that IL-4 did not accelerated disease recurrence.

As mentioned, the double graft protocol also provided for a second non-transduced islet graft to be transplanted in the event of disease recurrence following the first set of two grafts. For this second portion of the study only 3 of the original 5 mice/group were considered surgical technical successes as defined by attainment and maintenance of normal blood glucose over 5 d post transplant. In these animals disease recurrence was again no different between the three groups.

AdCMVmIL-10 Transplants: Since our preliminary experiments using AdCMVmIL-10 did not suggest any disease acceleration, the transplants with IL-10 followed a single graft protocol. NOD islets were transduced with either AdCMVmIL-10 or a control virus (AdCMVßGal) and placed under the kidney capsule of syngeneic diabetic recipients. Again no significant difference was seen between the two groups in terms of disease recurrence (Fig. 4-7 B).

Discussion

Our first objective was to evaluated the efficacy of the adenoviral expression system as a transfection technology for mouse pancreatic islet grafts. In particular, establishment of the in vivo expression of AdCMVßGal over time in the absence of disease was an essential prerequisite. In vivo expression on the order of several weeks would permit the evaluation of potential immune modulators expressed from transduced islets in the diabetic NOD transplant model. These results demonstrate that adenoviral vectors are an effective method for gene transduction and expression in islets both for in vitro and in vivo applications. Our results for AdCMVßGal transduced Balb/c islets are similar to those reported previously (Csete et al., 1995) but differ in some important aspects.

Our transfection efficiency was 51 % at an estimate MOI of 2.5, versus the 27 % at an MOI of 10 reported by Csete *et al.* (Csete et al., 1995). This discrepancy may be accounted for by the different islet isolation procedures or by culturing of islets at 37° C prior to infection in the previous study (Csete et al., 1995). As mentioned, our experience was that overnight culture greatly reduced efficiency of transduction. Liberation of islets from the exocrine tissue of the pancreas with collagenase reduces the collagen layer that otherwise surrounds the islet. In tissue culture at 37° C, islets will rapidly synthesize new collagen (R. Rajotte, unpublished results). We speculate that restoration of the collagen layer may be a factor in reducing the transfection efficiency of islets when they are cultured overnight prior to infection. A collagen layer may inhibit either receptor binding or internalization of the virus. If correct, this hypothesis may also provide an explanation for why some islets in a preparation seem not to be transducible by adenovirus. Differential exposure to collagenase during the isolation procedure, may result in variable collagen densities on individual islets which in turn could affect their susceptibility to adenoviral infection.

With in vivo expression of AdCMVßGal transduced and transplanted islets the longest time point previously reported for the detection of reporter gene expression is 1 month (Csete et al., 1995). Also, these transplant recipients maintained a very high "normal" blood glucose, equivalent to 11 mM. As evident from our work, true "normal" blood glucose levels are achievable with adenovirally transduced islet grafts. Given that the in vitro function of the islets in the work by Csete *et al.* (Csete et al., 1995) is unaffected by viral transduction, the higher blood glucose levels they report may simply be the result of the smaller islet mass used for the grafts (200 islets) relative to that reported here (400-500 islets).

The mononuclear cell infiltrate and the loss of ß galactosidase positive islet cells over time (Fig. 4-4), are consistent with the known potential of adenoviral vectors to induce an immune response which is attributed to the low level expression of adenoviral genes (Trembleau et al., 1995; Muruve et al., 1997) and ß galactosidase itself (Michou et al., 1997). The presence of ß galactosidase in grafts at either 30 days (Csete et al., 1995) or the 55 days seen in our work is 2 to 10-times longer than that seen with adenoviral expression in tissues such as liver (Shaked et al., 1994). It should be noted that the ß cells of a syngeneic islet graft in a diabetic NOD recipient are typically destroyed within 15 to 20 d. The in vivo expression seen with AdCMVßGal with transplanted Balb/c islets implies that an adequate expression window is provided to evaluate the autoimmune inhibitory potential of Th_2 cytokines in the diabetic NOD transplant model. Our results also suggest that in combination with tolerization protocols, such as the intrathymic injection of adenovirus (DeMatteo et al., 1995), much longer term expression from islets in vivo may be achievable with adenoviral constructs.

Even though adenoviruses are a highly efficient means of gene transfer into islets and do not appear to affect normal islet function, at least by the parameters reported here, the expression of either IL-4 or IL-10 does not provide any protection to the transduced graft in the face of an established autoimmune process. The picture that has emerged with respect to IL-4 and IL-10 administration in the NOD mouse is that timing and in some instances point of delivery are critical to the outcome of disease progression. Either of these two cytokines will prevent disease development when supplied systemically, well in advance of clear pathology (Rapoport et al., 1993; Pennline et al., 1994). This is also true of IL-4 (Mueller et al., 1996) but not IL-10 (Wogensen et al., 1994; Moritani et al., 1994) when supplied as a transgene expressed directly from the islet. However, once the disease process has progressed to a pathogenic state and activated islet specific T cells with cytotoxic potential, these cytokines do not appear to have any beneficial effect. The results reported here are consistent with those of others (Rabinovitch et al., 1995; Mueller et al., 1996; Wogensen et al., 1994; Moritani et al., 1994) in that beyond an early intervention window, neither IL-4 or IL-10 is capable of preventing the ultimate destruction of ß cells regardless of the method or site of delivery. The one notable exception to this generalization is that reported by Mueller *et al.* (Mueller et al., 1996) in which transgenic NOD mice expressing IL-4 from the islets were resistant to disease when challenged with syngeneic spleen cells from a fully diabetic donor.

With local adenoviral expression of IL-4, the dose of the cytokine over a 100 fold range did not seem to alter the outcome. It is difficult to make direct comparisons with respect to concentrations of cytokine between laboratories. However, the IL-4 production seen with the two viral doses in this study essentially bracket that reported in the NOD IL-4 transgenic work where the IL-4 expressing islets were transplanted into fully diabetic syngeneic mice (Mueller et al., 1996). Therefore, the two systems provide essentially a three point dose curve and have produced similar results in terms of the disease

recurrence/transplant model. It should also be noted that in spite of our concerns with respect to the potential rapid (48 h) destruction of cells expressing IL-4 seen in the tumor models (Tepper et al., 1992), such rapid destruction was not observed in either the islet transgenic studies with IL-4 or in our own work.

The acceleration of disease seen with IL-10 when present as islet cell expressed transgene (Wogensen et al., 1994; Moritani et al., 1994) was not evident in our work. However, the disease recurrence kinetics seen in both the control and IL-10 adenoviral transduced groups may not have provided sufficient time for such an observation.

An additional complication that must be considered when attempting to interpret the effect of either IL-4 or IL-10 in the context of our experimental system, is the background immunogenicity of the adenoviral vectors themselves. Since our results are consistent with observations in related models not involving adenovirus, and the vectors were controlled for in our experiments, we feel that our data does reflect the immuno-modulatory potential of IL-4 and IL-10 for disease recurrence in NOD mice. Further, a related study suggests that if there is a masking of the immuno-modulation of these cytokines, the real potential may not be that dramatic. When an adenoviral vector expressing an IL-12 antagonist (IL-12 p40 homodimer) was used to transduce islets in a transplant model equivalent to ours, average disease recurrence was delayed by 87 days (Yasuda et al., 1998). While these results may also represent an underestimate of the potential of p40 homodimer in this system, it does suggest that it is possible to over come the influence of the immunogenicity of the viral vector.

Given the results obtained by us and others (described above), and in particular considering the observation that an islet specific Th_2 cell line secreting IL-4 and IL-10 failed to prevent disease in the presence of an activated Th_1 diabetogenic T cell line (Katz et

al., 1995), it is doubtful that the combination of IL-4 and IL-10 would be any more likely to prevent disease recurrence in this model. So what other cytokines or potential combinations of these molecules could be considered? Broadening the Th_1 "suppressive" potential of IL-4 and IL-10 with the inclusion of TGFß is one possibility. TGFß has been shown to synergize with IL-4 and IL-10 to inhibit nitric oxide production and macrophage cytotoxic activity (Oswald et al., 1992). Also at least one of the viral forms of IL-10 as well as TGFß have shown promise in prevention of allograft rejection (Qin et al., 1995; Qin et al., 1996) and may have similar potential in suppression of the autoimmune process seen in IDDM.

Adenovirus vectors are indeed a very efficient means of gene transfer into mouse islets and even in the absence of tolerization protocols provide a medium term expression potential in vivo. However, IL-4 or IL-10 expressed in this context had no inhibitory effect on the autoimmune process in this disease recurrence model.

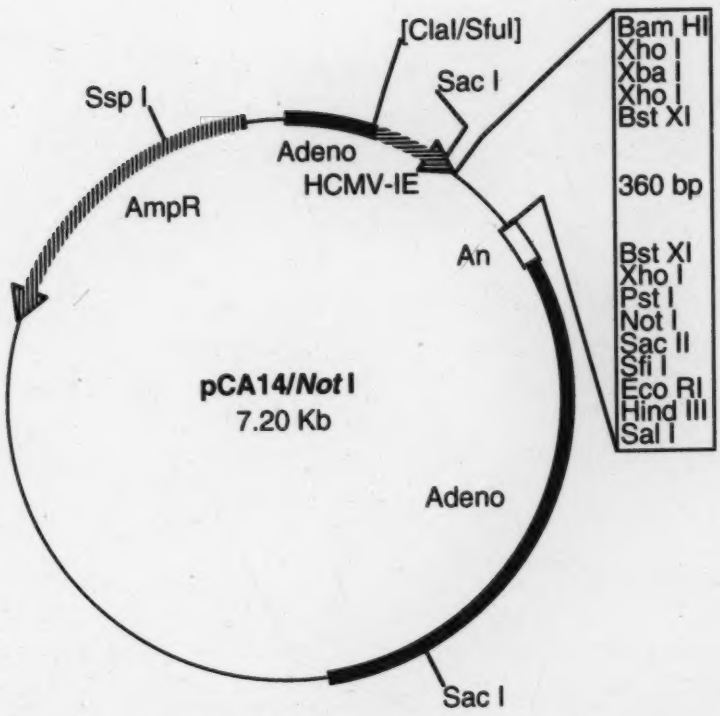

FIG. 4-1. Map of pCA14/*Not*I, a vector for the creation of recombinant adenoviruses. The vector pCA14 was modified by the insertion of the *Xba*I/*Eco*RI fragment from the polylinker of pJFE14 (Elliott et al., 1990) to create pCA14/NotI. Features of this vector include: adenoviral DNA sequences for homologous recombination (Adeno); the human CMV IE1 promoter (HCMV-IE); the pJFE14 polylinker (boxed text); a polyadenylation signal (An); and an antibiotic resistance marker (AmpR) for growth of the plasmid in *E. coli* under ampicillin selective conditions.

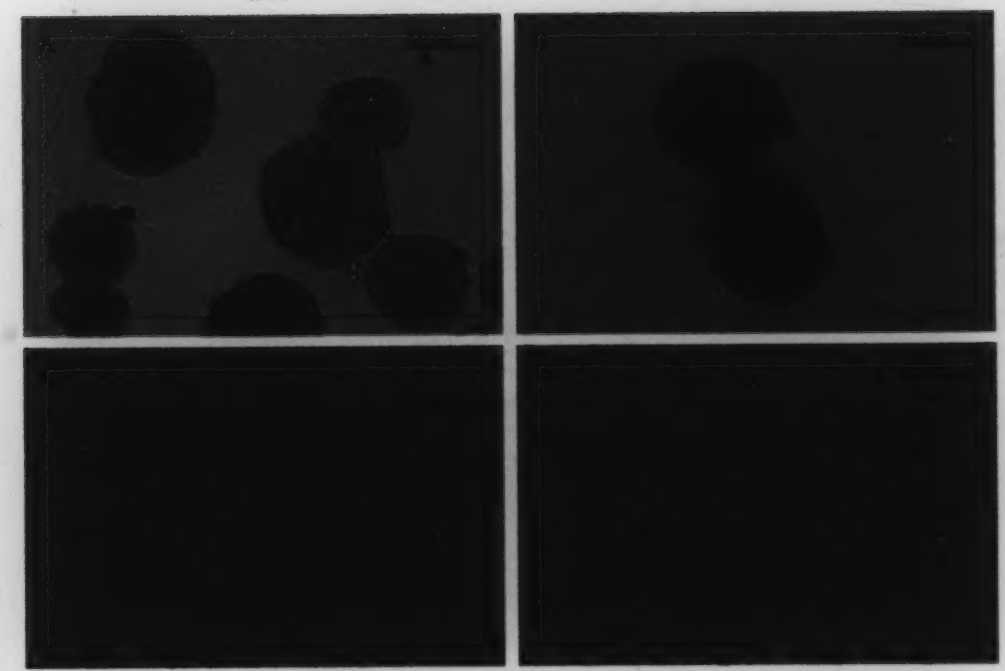

FIG. 4-2. Photomicrographs of control and AdCMVßGal transduced NOD islets before and after dissociation. Control islets after staining (A), dissociated control islet cells (C), transduced islets (B), dissociated transduced islets (D). Isolated Balb/c islets were incubated for 1 hour with 2.5 x 10^3 plaque forming units (PFU)/islet of AdCMVßGal. Free virus was removed with three washes and the islets were incubated at 37° C for 48 hours prior to staining for the presence of ß-galactosidase (18 hour incubation at 37° C in the presence of X-GAL). Single cell suspensions were prepared with trypsin and EDTA from transduced and control islets following the 48 hour incubation and then stained.

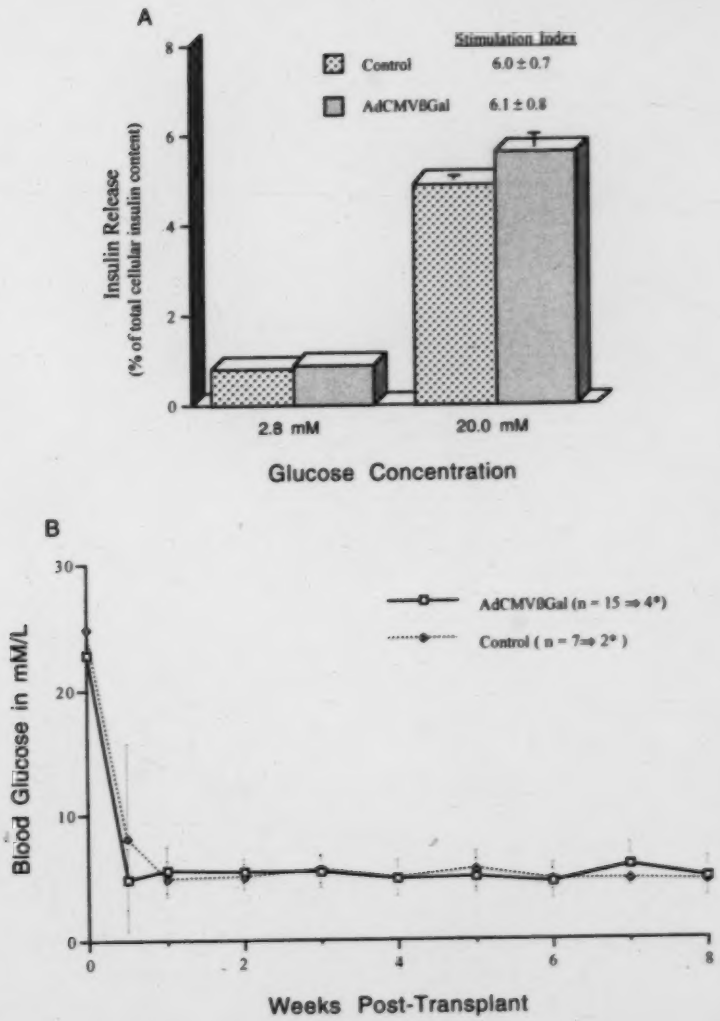

FIG. 4-3. In vitro and in vivo function of control and transduced (AdCMVßGal) Balb/c islets. A) 120 min in vitro static glucose stimulated insulin release assay conducted 48 h post transduction (2.5 x 10^3 PFU/islet). Values represent an average ± SD from 4 independent isolations and are expressed as percent release, with the associated stimulation indices. B) Non-fasting blood glucose levels for alloxan induced diabetic Balb/c mice with either control or AdCMVßGal transduced syngeneic islet grafts, transplanted under the kidney capsule.

*The n in both groups decreases over time since nephrectomies were performed over the 8 week study to confirm graft dependence for glucose normalization and examine expression of ß galactosidase.

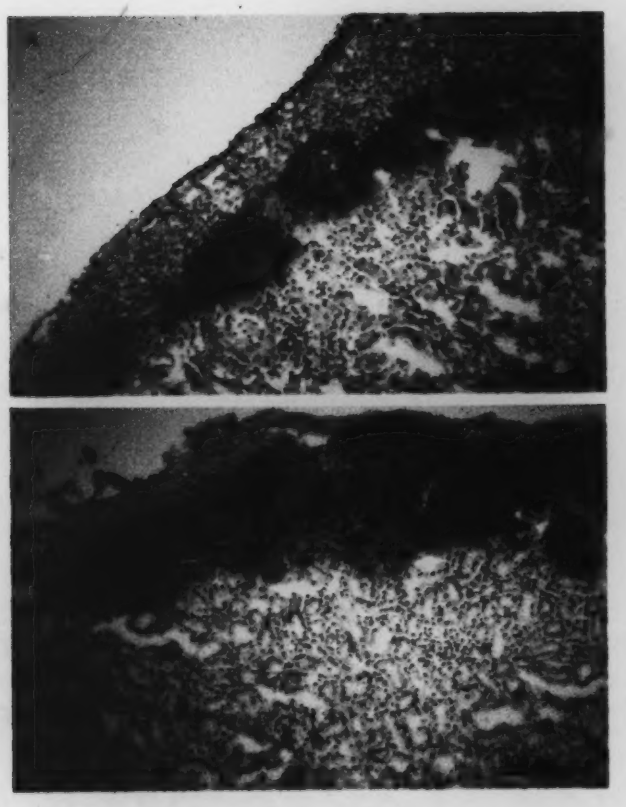

FIG. 4-4. Detection of ß-glactosidase over time in representative cryosections from AdCMVßGal-transduced Balb/c islet grafts. Cryosections from transduced grafts ($2.5x10^3$ PFU/islet) were removed on day 23 (A) or day 55 (B) after transplantation. Cryosections (6 μm) were stained with X-GAL, immunoperoxidase-stained for insulin, and then counterstained with hemotoxylin.

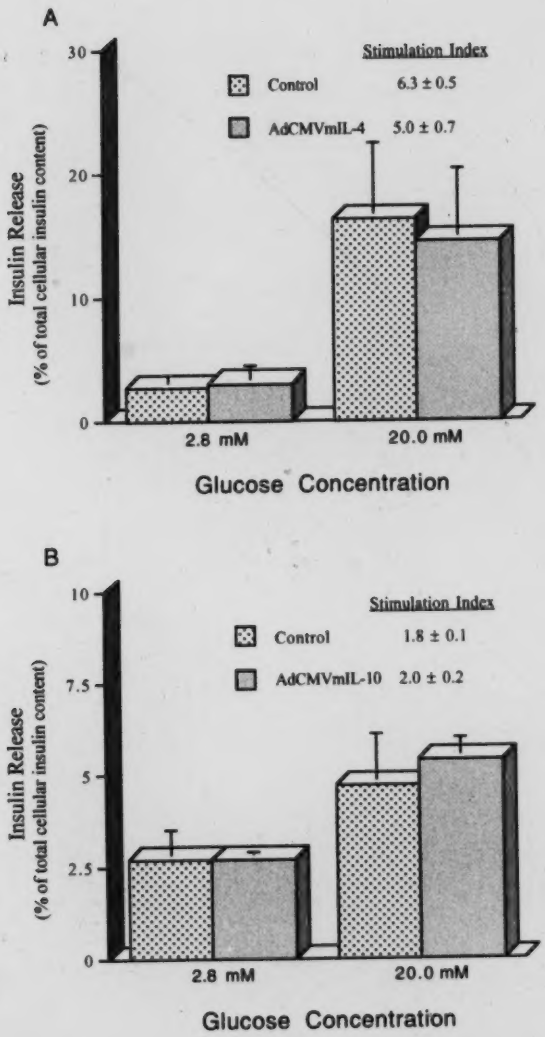

FIG. 4-5. Static glucose stimulated insulin release assays with NOD islets transduced with either IL-4 or IL-10 expressing adenoviral constructs. A) 120 min assay conducted 48 hours post-transduction with AdCMVmIL-4 at 2.5 x 10^3 PFU/islet versus a non-transduced control. B) Non-transduced control versus AdCMVmIL-10 transduced islets (2.5 x 10^3 PFU/islet) as above. Values represent an average ± SD from 3 independent isolations for each cytokine and are expressed as in Figure 1 A.

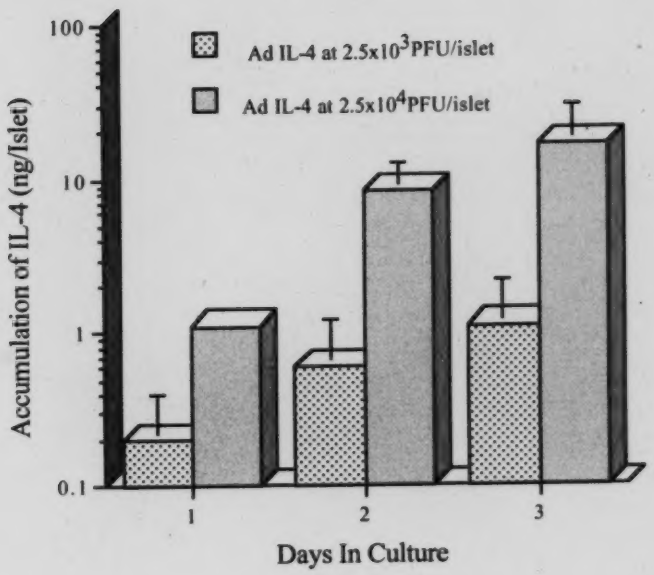

FIG. 4-6. IL-4 expression from Balb/c islets transduced with AdCMVmIL-4 as a function of time and dose of virus. Freshly isolated islets were transduced with AdCMVmIL-4 at 2.5 x 10^3 PFU/islet or 2.5 x 10^4 PFU/islet as indicated and placed into tissue culture. Values represent a mean ± SD from two independent isolations.

Table 4-1. In Vitro IL-4 and IL-10 Production From Adenoviral Transduced Pancreatic Islets

Cytokine	Strain	No. of Isolations	No. of Samples (n)	Range (ng/Islet)	Average (ng/Islet)
Il-4	Balb/c	3	4	0.11-0.80	0.39 ± 0.19
	NOD	4	6	0.11-0.31	0.21 ± 0.03
IL-10	Balb/c	2	5	0.08-0.64	0.29 ± 0.12
	NOD	2	2	0.05-0.07	0.06 ± 0.01

Values are listed as ranges and means ± SE for n replicates 48 hours post infection.
Freshly isolated islets were transduced at 2.5 x 10^3 PFU/islet.

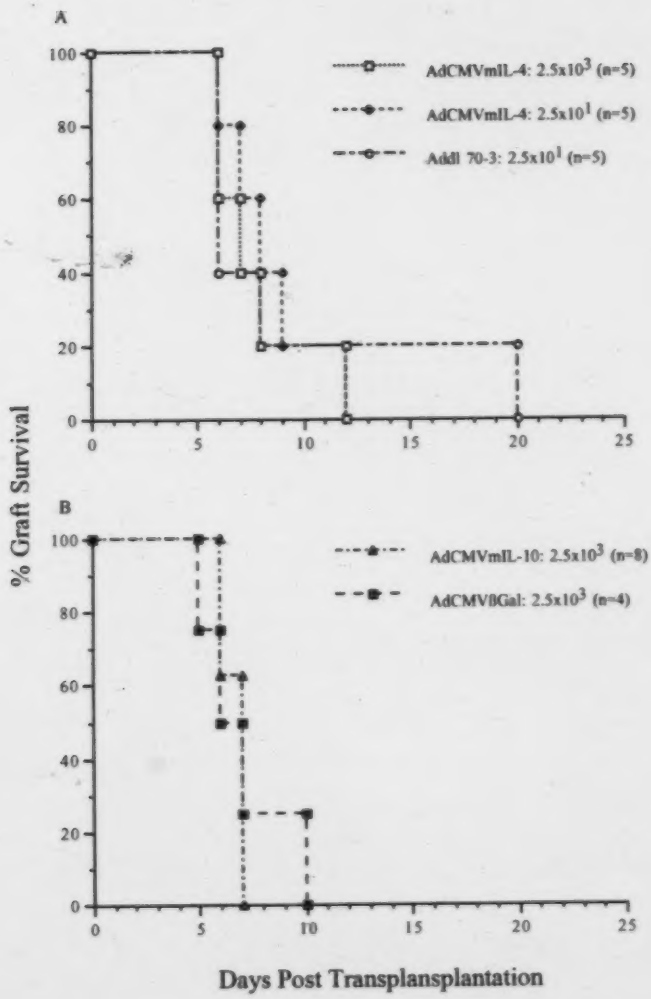

FIG. 4-7. Graft survival over time of cytokine adenoviral transduced NOD islet grafts transplanted into diabetic syngeneic recipients. A) AdCMVmIL-4 at two virus doses was used (25 PFU/islet and 2.5 x10³ PFU/islet) with the lower of the two doses also employed for the control virus, Addl 70-3. Diabetic NOD female recipients received a 400 islet syngeneic graft in each of its kidneys. One of each pair of islet grafts was non transduced. B) Control (AdCMVßGal) and AdCMVmIL-10 infected NOD islets (2.5 x10³ PFU/islet) in groups of 500 were transplanted into fully diabetic syngeneic female recipients.

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Chapter 5

General Discussion

Insights and contributions with respect to ecGAD: Irrespective of the conclusions regarding the antigenic relationship between ecGAD and mGAD, the cloning, sequencing and genetic localization of two homologous genes encoding ecGAD was an important contribution to bacterial genetics and physiology. As is outlined in Chapter 2, at the time we published our results the existence of a second highly similar *gad* sequence was a novel finding and the clones and antibodies we generated have proved to be a valuable resource to a number of laboratories (e.g. (Altuvia et al., 1997)). In addition, the localization of the two *gad* genes on the *E. coli* chromosome, each with a distinct DNA sequence, clarified considerable confusion with respect to these sequences. These efforts also proved a valuable lesson in both the power and pitfalls of PCR cloning strategies.

Both groups involved in these cloning efforts anticipated a single gene encoding ecGAD and both utilized PCR. Our concern over the error rate associated with *Taq* polymerase (the only PCR enzyme available at the time) encouraged us to use PCR to generate a probe which we would then utilize to isolate clones from a genomic library to avoid errors. However, the other group used PCR to generate their clones directly. Although, we did rely on the direct sequence of the original 470 bp PCR product to confirm the identity of our probe, because of the statistical nature of the data generated in a sequence reaction (where each band on a gel represents thousands of molecules), errors in the PCR product are not detectable and do not interfere with the interpretation of the data. However, when one clones isolated fragments of a PCR product, any errors that a given DNA molecule contains become the sequence. The case with *E. coli gad* was further complicated by the fact that there were two genomic sequences of identical length which

could not have been distinguished based on size alone when PCR products were analyzed with gel electrophoresis.

We had been alerted to the possibility of gene duplication by running an extensive series of restriction digests and Southern blots which revealed a double banding pattern in many of the digests (Fig. 2-5) suggesting the two genes. It should also be mentioned that coming to this project we were more familiar with eukaryotic genomes, where gene duplication is relatively common, and we were not aware of the comparative rarity of such highly similar sequences in prokaryotes. This reverse bias allowed us to readily accept the possibility of two genes and design a strategy to clone both sequences. A number of bacterial genomes have been sequenced recently including *E. coli* (Blattner et al., 1997). I'm pleased to report that both our sequences and their locations on the *E. coli* chromosome were confirmed.

Since our interest in these *gad* genes was immunological, we did not pursue the question raised with respect to the significance of these genes in *E. coli* but others have. Although, the role of ecGAD and the significance of two such similar genes is still not clear, it has been shown that each gene alone can encode an enzymatically active hexamer composed of three homodimers, and GADα and GADβ seem to be equivalent in terms of their biochemical properties (De Biase et al., 1996). Additional work by a separate group (using an anti-ecGAD Ab that I provided) has identified ecGAD as one of a number of enzymes that are induced by oxidative stress, which are regulated by a novel small RNA molecule denoted as OxyS RNA (Altuvia et al., 1997). In terms of the idea that the eukaryotic signaling molecule GABA is produced by *E. coli* and provides signals to the host immune system within the gut (or elsewhere), this remains in the realm of intriguing speculation. While there is at least one report of bacterial production of GABA (Minuk, 1986) it is not clear whether there are GABA receptors on immune cells, although this may

be a possibility that simply has not been explored (A. Bateson Department of Pharmacology, University of Alberta, personal communication).

Prevention of IDDM by ecGAD and CFA/BCG: As indicated in Chapter 3 we considered the response to ecGAD in the NOD mouse to be non-specific relative to islet antigens and hence the "protection" offered by ecGAD, the Stoffel control protein, and CFA all occur via similar mechanisms. As a result the previous published work with CFA provides some insight into the mechanisms involved beyond what was described in the Chapter 1.

Prevention of diabetes in NOD mice with CFA administration in the spontaneous model was initially ascribed to the induction of a non-specific suppressor activity identifiable in splenocytes (McInerney et al., 1991; Sadelain et al., 1990) and bone marrow (Sadelain et al., 1990). This phenomenon was characterized by its ability to down regulate spleen cell proliferation in response to Con A, anti-CD3, LPS or allogeneic antigens. The cells responsible for the suppression were described as Thy-1$^+$, non-adherent to nylon wool (Sadelain et al., 1990) as well as CD3$^-$, CD4$^-$, CD8$^-$, nonphagocytic, esterase negative, NK$^-$ and Mac-1$^+$ (McInerney et al., 1991). In fact all autoimmunity is not blocked in CFA treated mice, as indicated by the persistence of insulitis which results in diabetes following cyclophosphamide (CY) treatment (Qin et al., 1993). The same study also found that CFA treatment could up-regulate Con A responsiveness in thymocytes, that delayed diabetes onset could be transferred by Thy 1.2$^+$ or CD4$^+$ spleen cells, and that the protection offered to islets by CFA was slightly more stable than that induced with heat killed *M. tuberculosis* in the absence of IFA, or by live BCG vaccine administered as a foot pad (f.p.) injection.

The role of Mϕ appeared to be more complex. With Mac 1$^+$ cells obtained from spleens of CFA f.p. injected mice (McInerney et al., 1991) or live BCG i.v. vaccinated animals (Harada et al., 1990; Yagi et al., 1991b; Yagi et al., 1991a) these cells would transfer

protection whereas peritoneal Mac 1^+ cells offered little protection (Qin et al., 1993). It should also be noted that the protection observed with the Mac 1^+ transfers were free of insulitis in contrast to T cell enriched transfers (Yagi et al., 1991a). Further support for a Mac 1^+, class II^+, $B220^-$ cell involvement in disease prevention was provided by the observation that i.v. injection of heat killed BCG protected NOD mice, and this was associated with a two- to four-fold increase in antigen presenting capacity. However, this treatment also appeared to induce a SLE-like syndrome (Baxter et al., 1994). In contrast, such autoimmunity has not been reported in any f.p. injected CFA or BCG study even when it was specifically looked for (Shehadeh et al., 1994).

The potential involvement of Mϕ and up-regulation of Ag presentation is significant for two reasons. The first is the previously mentioned defect in NOD APC function in terms of depressed SMLR, antigen presentation, and cytokine secretion upon cell activation (Serreze and Leiter, 1988; Leiter and Serreze, 1992; Serreze et al., 1993a; Serreze et al., 1993b), in conjunction with the equally well established consequence in terms of immune suppression of CTL mediated responses with BCG and Freund-type adjuvants (Asherton and Allwood, 1971; Zola, 1972; Bennett et al., 1978). Both of these phenomena are potentially influenced by the initial up-regulation of Mϕ function by mycobacterium, which is associated with production of TNFα and IL-1ß (Zhang et al., 1993). In turn, these cytokines have both been implicated in diabetes prevention (Jacob et al., 1990; Grewal et al., 1996) and initiation (Rabinovitch, 1998). Additional evidence that these adjuvants are non-specifically suppressive with respect to autoimmunity comes from the EAE models that require immunization of susceptible mouse stains with MBP in CFA. Prior immunization with CFA, PPD or PT in the absence of MBP results in protection from EAE (Falk et al., 1969; Lisak and Zweiman, 1974; Ben-Nun et al., 1993). The known disease-suppressive mechanisms induced by CFA and BCG are probably only a subset of the complex interactions that result from generalized immune stimulation. However, not to lose sight of

the significance of these findings, it is important to remember that in the absence of any immune stimulation, at least some NOD colonies have all the necessary and sufficient predisposing conditions to develop 100 % incidence of diabetes in female mice.

As indicated in Chapter 1, the NOD mouse is in an immunologically dysregulated state with respect to APC function, cytokine networks and several other parameters (recently reviewed (Delovitch and Singh, 1997)). Non-specific stimulation with CFA or other microbial antigens in IFA (e.g. ecGAD or Stoffel protein) may not all be acting in precisely in the same fashion, but all may be resulting in at least a partial restoration of the immunological network that is otherwise dysfunctional. One of the downstream consequences of early activation events seen with microbial adjuvants is altered cytokine patterns in the islet infiltrating cells or in islet Ag specific T cells in the spleen. This was first described in kidney capsule "sentinel grafts" transplanted into CFA protected NOD mice. The infiltrates in protected grafts were shown to have a higher ratio of IL-4/IFNγ producing cells relative to grafts transplanted into diabetic animals, which had a low IL-4/IFNγ ratio (Shehadeh et al., 1993). Similarly, CFA and live BCG in the spontaneous disease model, and live BCG in the transplant model induce mGAD67 responsive spleen cells that produce IL-4 but no IFNγ, and these cells show reduced proliferation in response to mGAD67 (Qin et al., 1995). Also, early i.p. injection of CFA in the NOD mouse results in reduced IFNγ production from the islet infiltrating cells, but no increase in IL-4 levels (Rabinovitch et al., 1995).

Results compiled by our pathology collaborator on pooled tissues obtained from both CFA and live BCG protected grafts revealed an immunohistological picture compatible with the PCR results obtained in the NOD mouse (Halstensen et al., 1995). Two-color immunohistochemical staining identified the relative proportions of CD3, CD4, and CD8 T cells, B cells (B220+) and Mϕ (Mac 2+). Cytokine production was determined by in situ

immunostaining for IL-4, IL-5, IL-6, IL-10, GM-CSF, TNFα and IFNγ. Grafts removed from controls with recurrent diabetes had a dense intra-islet infiltrate consisting of $CD3^+$ cells with more $CD4^+$ cells than $CD8^+$, numerous Mac 2^+ cells and scattered $B220^+$ B cells. Both T cell subsets infiltrated the graft. However, the CFA treated grafts had the typical peri-islet infiltrate with almost as many $B220^+$ cells as $CD3^+$ T cells, but few Mϕ. Cytokine expression was not different between the CFA protected and intra-islet infiltrated grafts with the exception of $IFN\gamma^+$ positive cells. These were greatly reduced in the CFA protected grafts as compared to grafts from diabetic animals. In the CFA protected grafts the median count for $IFN\gamma^+$ positive cells was 0 with a range of 0 to 2 per cross sectional area (c.c.a.), as compared to a median of 14 with a range of 8 to 34 cells per c.c.a. within the diabetic grafts. The increased proportion of $B220^+$ B cells and the lower proportion of $IFN\gamma^+$ cells in the CFA protected grafts is indicative of a down regulation of a Type 1 pattern. Similar results were found in terms of $IFN\gamma^+$ down regulation and an increase in $B220^+$ B cells in CFA treated NOD mice in a more definitive study (Suarez-Pinzon et al., 1996). However, in this latter study $IL\text{-}4^+$ T cells in the islet was seen to increase in the CFA group but not in the spleen for same group. From these data it is not possible to know the cytokine production per cell, only the frequency of positive cells.

However, if the situation in this experiment is similar to previous work in the NOD mouse, even the level of IL-4 production in the protected grafts is largely unchanged as determined by semi-quantitative PCR (Rabinovitch et al., 1995). With respect to IFNγ, a few points should be mentioned. Correlation of the presence of the cytokine with the presence of a particular cell type does not necessarily allow one to draw conclusions about cause and effect. With the results observed with the IFNγ receptor KO described in Chapter 1, the picture with respect to the role of this cytokine has become clearer. However, there are still confounding results that show a delay in disease progression when IFNγ and TNFα are administered systemically (Campbell et al., 1991). This latter

situation likely reflects the consequence of generalized immune stimulation, and unlike the results seen when IL-4 was administered or expressed as a transgene, the state of "protection" induced by IFNγ plus TNFα is not as robust, and not necessarily permanent (Campbell et al., 1991).

The extent and degree to which non-specific immune stimulation results in delay or prevention of disease in the NOD seems to be a function of the immunogenicity of the agent and the timing of its administration. In the spontaneous model, early administration of a potent immunogen can potentially influence the lineage development of effector and regulatory immune cells prior to the development and activation of pathogenic ß cell specific T cells. This seems to result in a relatively stable situation in which the competition between non-destructive regulatory cells and pathogenic effectors is in favour of disease prevention.

At disease onset, fully activated islet specific effectors have destroyed approximately 80% of the ß cell mass, yet a potent intervention even at this late stage can reverse the diabetes in a majority of animals. This has been demonstrated with anti-CD3 treatment in CY induced diabetes. When the anti-CD3 antibody was given within 1 week of disease onset complete remission was seen in 64 to 80% of animals, and those that did not normalize their blood glucose did so following an islet transplant (Chatenoud et al., 1994). This level of response can be contrasted to the decreasing effectiveness of CFA as diabetes onset approaches (Qin et al., 1993). My own unpublished results with live BCG administration at disease onset showed clearly that no remission from disease could be induced (data not shown), which is not unexpected given the mechanisms likely to be at work. The optimism with respect to clinical trials with BCG for early diagnosed IDDM patients (Shehadeh et al., 1994) was clearly not justified (Elliott et al., 1998).

The last situation to attempt to account for, at least in a theoretical way, is the delay in disease recurrence observed with microbial adjuvants given in the diabetic transplant model and the diabetic spleen cell challenge assays. These are the most puzzling of the phenomenon associated with these "protection" experiments and for which the least is known. Presumably the mechanisms that are active at disease onset, are also operational at the time of transplant and immunization. Yet in the spontaneous disease model, if CFA (or BCG) is administered at onset it has little effect, while it will have a significant impact on the delay of disease recurrence if administered at the time of transplant.

As has been pointed out, the timing of the immunizations for protection in either the transplant or diabetic spleen cell transfer is critical and administration of CFA must be at the time of transplant/transfer, or alternately it can be delayed by at most 24 hr (Ulaeto et al., 1992). One additional consideration in both these models is that the islet reactive lymphocytes must make their way to the graft or pancreas under the influence of the adjuvant. I.v. injection of CFA profoundly up-regulates lymphocyte trafficking and perhaps the same is true to a lesser extent with f.p. or other routes of administration. The micro-environments that lymphocytes migrate through in the context of the "cytokine burst" and potential up-regulation of cell surface molecules as well may explain the delay seen in an adjuvant treated diabetic mouse. This perhaps could be considered a distraction/suppression model of protection. Distraction in this context refers to both the potential for increased lymphocyte migration and the fact that the adjuvant is typically injected into a site distal to the graft. No one would suggest co-administering CFA under the kidney capsule with the islet graft expecting a favorable outcome in terms of the transplant survival. It should also be noted that in contrast to the long lasting and reproducible protection that results from early immunization of NOD mice with CFA, BCG and ecGAD, prevention of disease recurrence is a more ephemeral phenomenon (in our experience). In fact, other transplant centers have also had difficulty achieving long term

(>80 days) graft protection in the CFA disease recurrence model (Ron Gill personal communication).

Given that we were unable to establish an antigenic link between mGAD, rGAD or islets, I suggest that the mechanisms involved in the prevention of diabetes observed with mGAD are most likely similar to those seen with other non-specific agents such as CFA and related microbial adjuvants. Since cytokines had been shown to play a key role in microbial adjuvant non-specific immune stimulation as well as disease pathogenesis, I next undertook an investigation of the role that cytokines might have in disease prevention in a gene therapy transplant model of disease recurrence.

Adenoviral transduction of islets with IL-4 and IL-10: Our results obtained with these cytokines in the transplant model were in agreement with the observations in related transgenic experiments done by others (Mueller et al., 1996) and with the inability of either cytokine to down regulate strong ongoing autoimmune responses. However, one of the more interesting results obtained in the transgenic experiments was the ability to prevent ß cell destruction when IL-4-RIP transgenics were challenged with wild-type NOD diabetic spleen cells, but not when the IL-4 expressing transgenic islets were transplanted into diabetic NOD animals (the latter situation being comparable to our transplants). With the transgenic transplants the authors felt that the trauma associated with the surgery may have been responsible for the different outcomes (i.e. transplant versus spleen cell challenge). This may well be the case given what is known to happen when a CD8 H-2K^d restricted islet specific T cell clone is co-transferred with islet MHC compatible grafts as potential targets. If the CD8 clones were transferred within 2-3 days of surgical implantation of the graft, then ß cell destruction would ensue (Utsugi et al., 1996). If however, the grafts were allowed to "heal" for a period of 8 days or more prior to T cell transfer, the CD8 clones were unable to home and establish insulitis let alone cause disease. Unfortunately,

comparable results for a CD4 clone were not presented. If it were possible to pharmacological suppress the β cell attack for even a short period of 8 to 10 days, it may be possible to observe graft protection with transplanted IL-4 transgenic islet grafts (or even the adenoviral transduced islets). However, it is not clear that any pharmacological treatment short of anti-CD8 would be able to achieve sufficient suppression, and as mentioned that in itself will prevent disease recurrence in the NOD.

Back to the original paradox: Why is the islet attacked? It is clear that MHC is an essential predisposing factor for autoimmune diabetes and this is illustrated in recent TCR transgenic work demonstrating negative selection occurring when a disease resistant MHC molecule is introduced (Schmidt et al., 1997). This together with the observation that I-A^{g7} is a poor presenter of peptide suggests that a diabetogenic MHC establishes a situation in which there is a higher than usual frequency of autoreactive T cells allowed into the periphery. In the case of the NOD (and possibly the human) this is combined with defective APC function and defective peripheral tolerance induction. However, the NOD does not show widespread autoimmunity, but rather a tissue restricted pattern which always includes the islets and salivary glands and may involve other endocrine tissues. It seems clear that the infiltrates in these various tissues are not seeing related antigens. What then recruits the first APCs to the site of the islet or the salivary gland to initiate an inflammation? What we know of normal APC function would suggest it is not necessarily the Ag which is important. Dendritic cells and other APCs sample their environment as they move through tissues. They will only begin to home or stick to a site when they are activated, and this is not affected by antigen but by proinflammatory molecules such as chemokines or other stress related factors due to tissue damage or microbial activators resulting from infection (e.g. bacterial or viral DNA, RNA, LPS etc.).

If these are the only conditions that will recruit APCs then it implies that insulitis is initiated due to spontaneous islet degeneration (for which I am not aware that there is any strong evidence) or to viral infection, which might possibly be an endogenous retrovirus for which there is some evidence. However, in the NOD it must be an endogenous virus since females will develop IDD % disease when raised under gnotobiotic conditions, which dictates that all the etiological factors are present within the mouse itself. There is however one other possibility.

As mentioned, all the targets of autoimmune disease are endocrine tissues which are secreting a number of regulatory molecules. It may be that either an inappropriate signal is being transmitted to which dendritic cells/APCs are sensitive, or normal signals are being secreted but due to a defect or defects in the APC the signal is being misinterpreted. These alternatives of course are not mutually exclusive and in fact could combine with viral factors which could be induced by the presence of partially activated dendritic cells as the infiltrate builds (Tsumara et al., 1994). The process is a slow one which suggests that if these mechanisms are at work, this inappropriate exchange of signals is just exceeding a threshold of the APCs and therefore, the process may be difficult to quantify. However, the search for signaling molecules could be aided by the fact that the two primary sites of inflammation might possibly share a similar signaling defect. It is also instructive to remember the novel T cell subset with elevated insulin receptor density that marked them as highly diabetogenic (McInerney et al., 1996). The type of chemotaxis ascribed to insulin gradients that was suggested in this recent work, may be a model of the process described above.

Ag in this model is still critical since full activation of T cells will require Ag and here again MHC will exert its influence by selecting proteins and epitopes that can be presented efficiently, which accounts for a limited initial array of antigens and epitopes. Availability

ably also a factor. A tentative sequence of events that fits with the genic evidence is the following.

ritic cells are inappropriately recruited to the islet and further activated fic signals. This seems to be a slow process in the NOD mouse, ly three weeks to become obvious histologically. (It is intriguing to c interval is the same period over which the consequence of TNFα n diabetogenic (Yang et al., 1994) to protective in the NOD mouse As partially activated dendritic cells migrate back and forth between ining lymph nodes, they slowly recruit CD8 T cells (Jarpe et al., d B cells to the site (Jarpe et al., 1990; Jansen et al., 1994). With the riately activated Ag specific B cells, this could accelerate antigen er through specific killing (Fas presumably being critical but also Mϕ derived products (or both), ß cells would be destroyed. I would non-specific cytokine mediated killing would predominate initially and would only be late stage effector mechanisms, since these lethal nder stronger regulatory control. The ß cell may become the target its relative sensitivity to inflammatory factors and later due to Ag subsequently develops. Since peripheral tolerance is not completely this developing network of hostile cells is competing with an alternate h self tolerance. Unfortunately, this is typically a losing battle, since factors that would aid in the maintenance of tolerance are defective in resumably with a defined critical mass of infiltrating cells and no he process (i.e. immunostimulation), once a threshold of CD8 (or cells are activated the aggressive stage of disease sets in and massive curs. This is clearly a speculative model of disease initiation but han the antigen driven proposals that lack a triggering mechanism to

initiate the process. If this endocrine triggered immunological model has merit then it would have significant implications for the direction of future research and the quest for the "holy grail" would be over.

Future Directions

Gene therapy with pancreatic islets: One area of the work with the IL-4 and IL-10 adenovirus constructs that should be expanded, is the characterization and timing of the infiltrate at the islet that might be influenced by the cytokines being expressed. In the series of experiments reported here, grafts were only taken at the time of disease recurrence and a detailed characterization was not undertaken. Additionally, while the AdCMVßGal construct confirmed the in vivo expression of a model recombinant gene product in the transduced islets, cytokine expression was not directly confirmed in vivo. This could be particularly important over time, since these cytokines are rapidly consumed and degraded in contrast to the relative stability of the ßGal molecule.

Although the specific cytokines used in these studies did not demonstrate any immune modulation, the vector system proved to be effective. Of the gene transfer technologies available for islets, the adenovirus system is amongst the most efficient. Adenoviral transduction efficiencies of islets in vitro reach can reach 100% and islets transduced with engineered adenoviruses express relatively high levels of recombinant proteins. The disadvantages of this system include the labor intensive process of creating recombinant clones, and the host immune response against a number of adenoviral proteins that are expressed at a low level in addition to the recombinant protein. The latter problem of immunogenicity has been addressed by generating new deletant recombinant adenoviruses that produce only recombinant proteins, minimizing the number of antigens for the host to mount an immune response to. Non-integrating viruses (e.g. adeno and adeno associated virus) have become one of the more popular gene transfer technologies in diabetes research, as well as in unrelated fields such as cancer vaccine development and several other gene therapeutic applications.

David Curiel (Curiel, 1994) has developed an adenovirus-mediated gene delivery method that circumvents the need to generate a recombinant adenovirus for each new DNA sequence that one wishes to express. By complexing plasmid DNA containing a target gene to the outside of the adenovirus protein capsid, the virus becomes a carrier for the DNA: i) providing entry into permissive cells, ii) promoting endosomal lysis, iii) and targeting to the construct to the nucleus. Adenoviral-plasmid conjugates can be made in minutes rather than the weeks typically required to generate adenoviral recombinants harboring target genes. Additional ligands can also be attached to the viral capsid to enable entry to cells that would normally not be tropic for the virus. Although this is an effective system for tissue culture, it is not appropriate for in vivo work. This is due to the fact that the complexes are not stable in high serum concentrations. Given the potential usefulness of this adaptation of the adenoviral transfection technology, improvements in the stability of the DNA/virus linkage could permit the use of these complexes in vivo. Regardless of the stability of the of the complexes, endotoxin free DNA must be used, since the viral capsid will traffic LPS (or other contaminants) into the cell just as efficiently. Endotoxin-adenoviral associations can lead to reduced transduction efficiencies or even the death of cells, with relatively low levels of LPS (Cotten et al., 1994).

While creating improved gene transfer technologies is an important consideration, determining which genes to transfer is of higher importance. In autoimmune diabetes one clear objective is to identify factors that will down-regulate activated CD4 and CD8 effectors. In vitro systems that mimic the ß cell assault, could facilitate the discovery of novel combinations of potential factors. Such a system have been developed by isolating islets associated lymphocytes and activating these cells with IL-2. This produces an activated cell suspension that is capable of rapid ß cell lysis within a mater of hours. This is clearly be a stringent system in which to examine modulation but if one were to succeed in this system, one might have a higher probability of successes in vivo.

Having such an in vitro system also allows one to begin examining questions of signaling as a consequence of the various manipulations. Such studies would add depth to our understanding of these interactions. Another principle advantage is that numerous combinations of molecules can be studied efficiently which is something that is not feasible in vivo. When secreted molecules are being examined, simple addition to the media would be adequate and where cell surface expression was required one would have to transfect the cells. This could involve any of the adenovirus systems discussed above amongst the many new transfection strategies that are currently being developed. Some of the more obvious cytokine combinations which could be examined in an in vitro system are mentioned at the conclusion of Chapter 4.

If one considers "expression" vehicles other then islet cells, in the attempted to modulate immune responses against the islet, then the B cell or other APCs could provide interesting possibilities. Engineering of Ig receptors on "APC" like cells to enhance islet antigen presentation but in association with "death" signals (such as Fas L) to delete autoreactive T cells. Methods have been developed to use an Ig that is specific for a protein tag to which Ag can be conjugated and thereby provide an "Ag specific" internalization and presentation, which can be superior to that observed in dendritic cells. Again the use of an in vivo model system would be useful to evaluate this strategy, designed to remove the T cell mediated ß cell destruction.

APC and islet interactions: Some brief comments on the potential for work in this area will conclude the thesis. The obvious first step is to determine if NOD dendritic cells (or Mϕ) and islets behave uniquely in terms of APC activation and chemotaxis. As noted previously, these effects are likely to be subtle, which will complicate the analysis. However, if such studies yield observations in support of the endocrine activation

hypothesis, then specific proteins and supernatant fractions could then be examined and these studies could be extended to the salivary glands which may provide additional insight. Molecular approaches such as differential display or subtraction cloning could be unitized to compare APCs or islets from NOD and other strains not susceptible to diabetes. Again the fact that the salivary gland may bear the same defect could aid this analysis.

Finally, an important point to make with respect to antigens in the context of this hypothesis is that even if the endocrine recruitment (activation) proposal can be confirmed, it will not diminish the requirement for future antigen research in diabetes. However, it may change the focus of some of this work. Rather than attempting to identify the triggering antigen, the objective could be to simply identify the most modulatory. Antigen modulation of the autoimmune response in diabetes and the study of autoantigens as markers of pathology are likely to remain as important aspect of diabetes research for the for the foreseeable future. This is likely to be the case even if new avenues of therapeutic intervention are identified with work such as that proposed here on dendritic cells.

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Appendix 1

Additional Methods

This appendix contains methods that are relevant to this thesis that the author was not directly responsible for and that are not published elsewhere.

Methods in Chapter 2:

Plating and probing the Kohara miniset library: 100 µl of 2xYT 10% glycerol was added to each well containing the ordered set of clones (in 96 well plates) and phage particles allowed to elute overnight at 4°C. Fresh lawns of *E. coli* NM 621 were poured onto 10 cm plates, and after 3 hours growth a 48-pin transfer apparatus was used to transfer a ≈ 2µl drop of phage particles from each well onto the bacterial lawns. These were grown until an array of large plaques had formed with the same geometry and corresponding to the original set of clones in the 96-well plates. The plaques were transferred to nitrocellulose filters and the filters baked, hybridized, and washed as described in Chapter 2.

Methods in Chapter 3:

GAD activity assay: The identity of the purified GAD protein was confirmed enzymatically with a radio labeled substrate and separation of the products based on charge (Dover and Halpern, 1972). Briefly, the modified protocol (D. Markland and J.F. Elliott unpublished results) involved the removal to cold substrate from the sample with a Centriom 30 in the presence of the enzyme buffer (5 mM EDTA, 0.1 mM 2-aminoethylisothiouronium bromide hydrobromide (AET; Sigma), 0.1 mM PMSF, 20 µM PLP). After washes and

reconstitution, 10 µl aliquots of sample were incubated with 9 µl of reaction buffer (50 mM K_2HPO_4 buffer pH 6.5, 0.2 mM PLP, 120 mM NaCl, 1 mM AET, 1 mM PMSF) and 1 µl of ^{14}C-glutamate (Amersham, Buckinghamshire, England; specific activity 59 mCi/mmol, or 50 µCi/ml) at 37° C for 4 h. The products of this reaction were separated on BioRad AG1X8 ion exchange resin contained within a 1.5 ml microfuge tube adapter (Millipore, Corp.), which permitted the neutral charged [^{14}C]-labeled GABA to elute after a brief spin (325xg) while retaining any negatively charged unconverted labeled glutamate. The eluted labeled GABA was then transferred into 5 ml of scintillation fluid and counted.

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Appendix 2

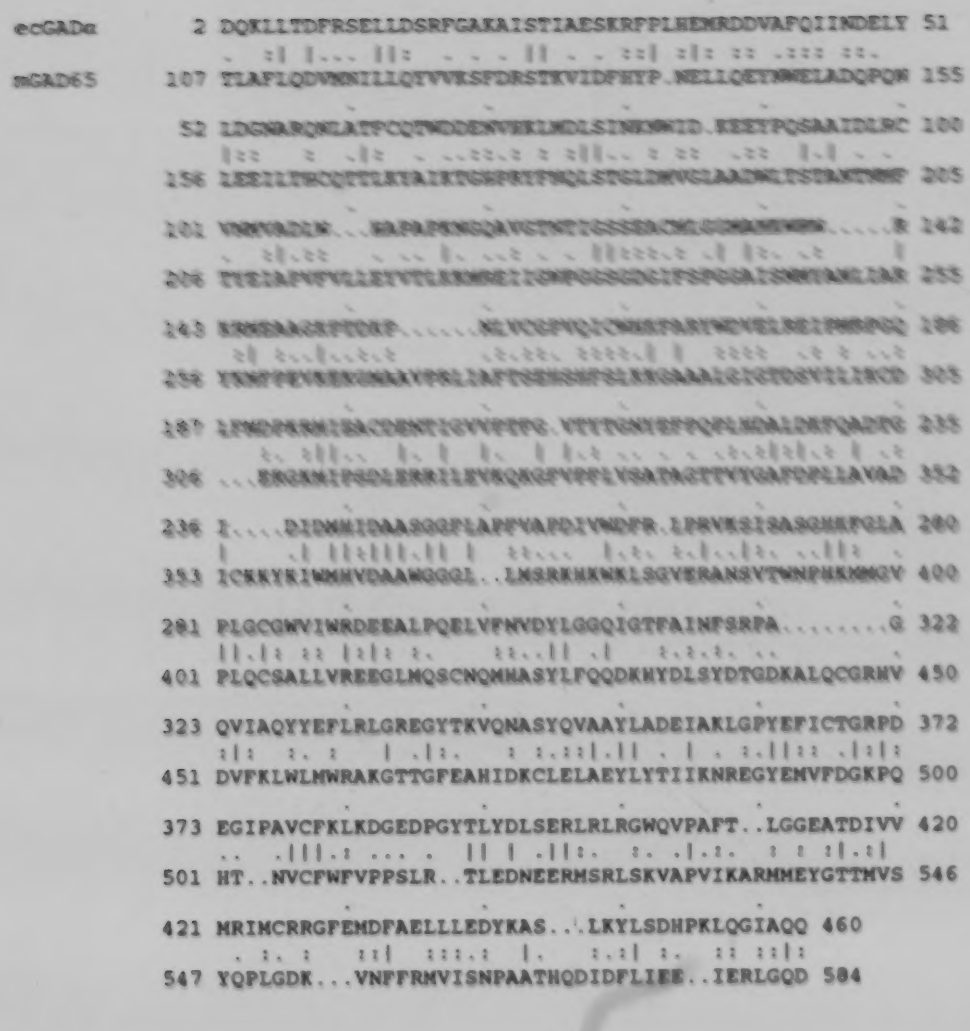

FIG. A-2-1. Best Fit alignment of ecGADα versus mGAD65 polypeptides.

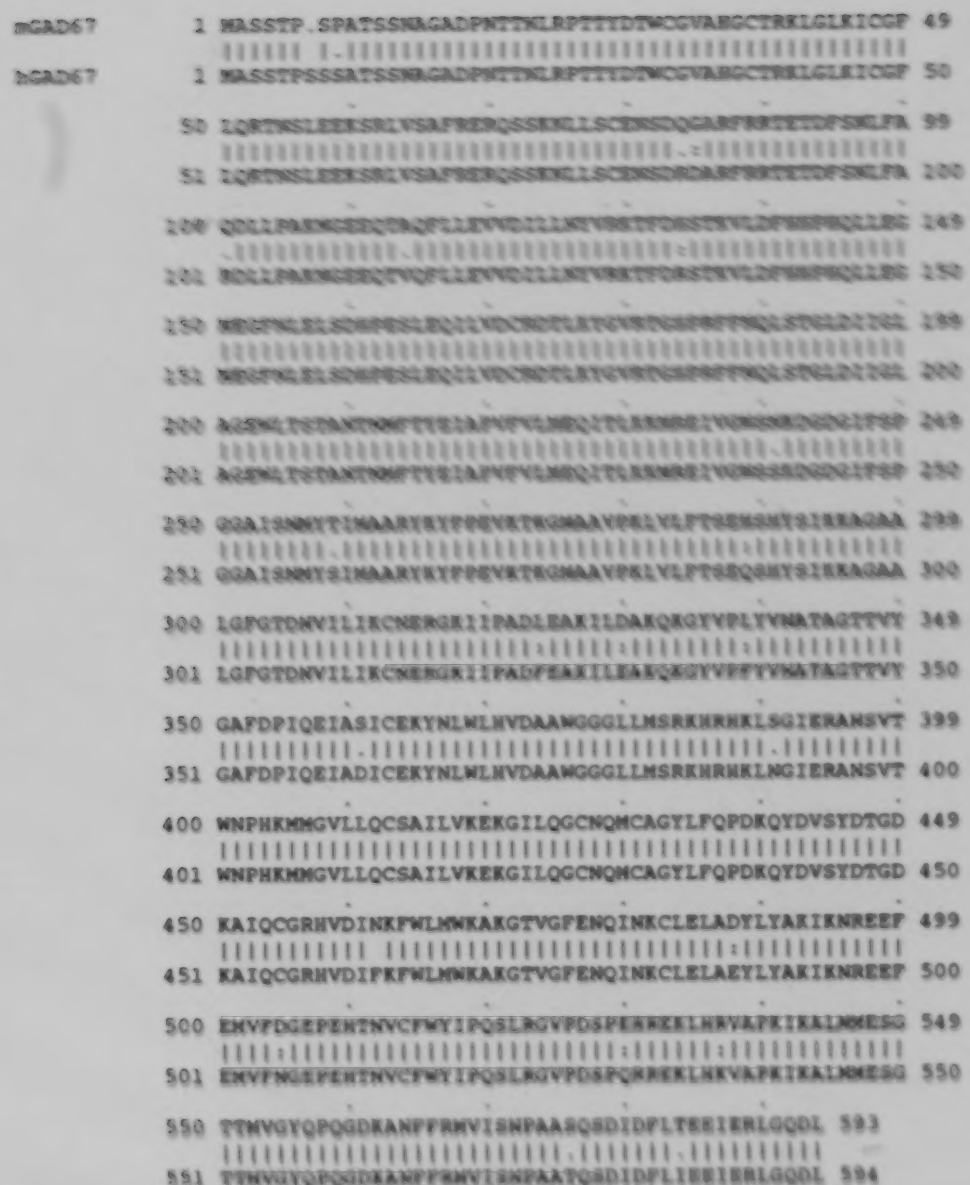

FIG. A-2-2. Best Fit alignment of mGAD67 versus hGAD67 polypeptides. This alignment indicated a 90 % identity and 98 % similarity between these two proteins.

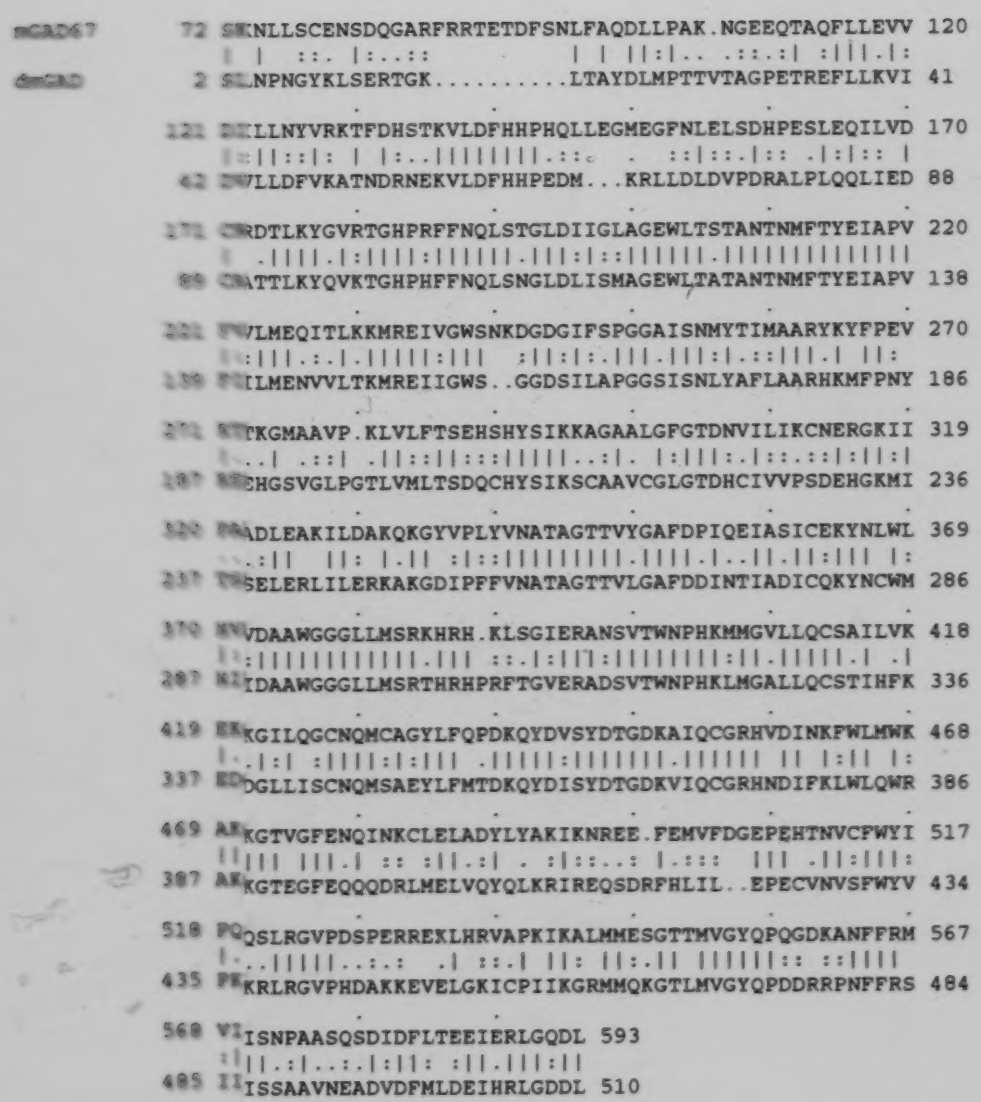

FIG. A-2-3. Best Fit alignment of mGAD67 versus dmGAD (*Drosophila melanogaster*) polypeptides. This alignment indicated a 56 % identity and 77 % similarity between these two proteins.

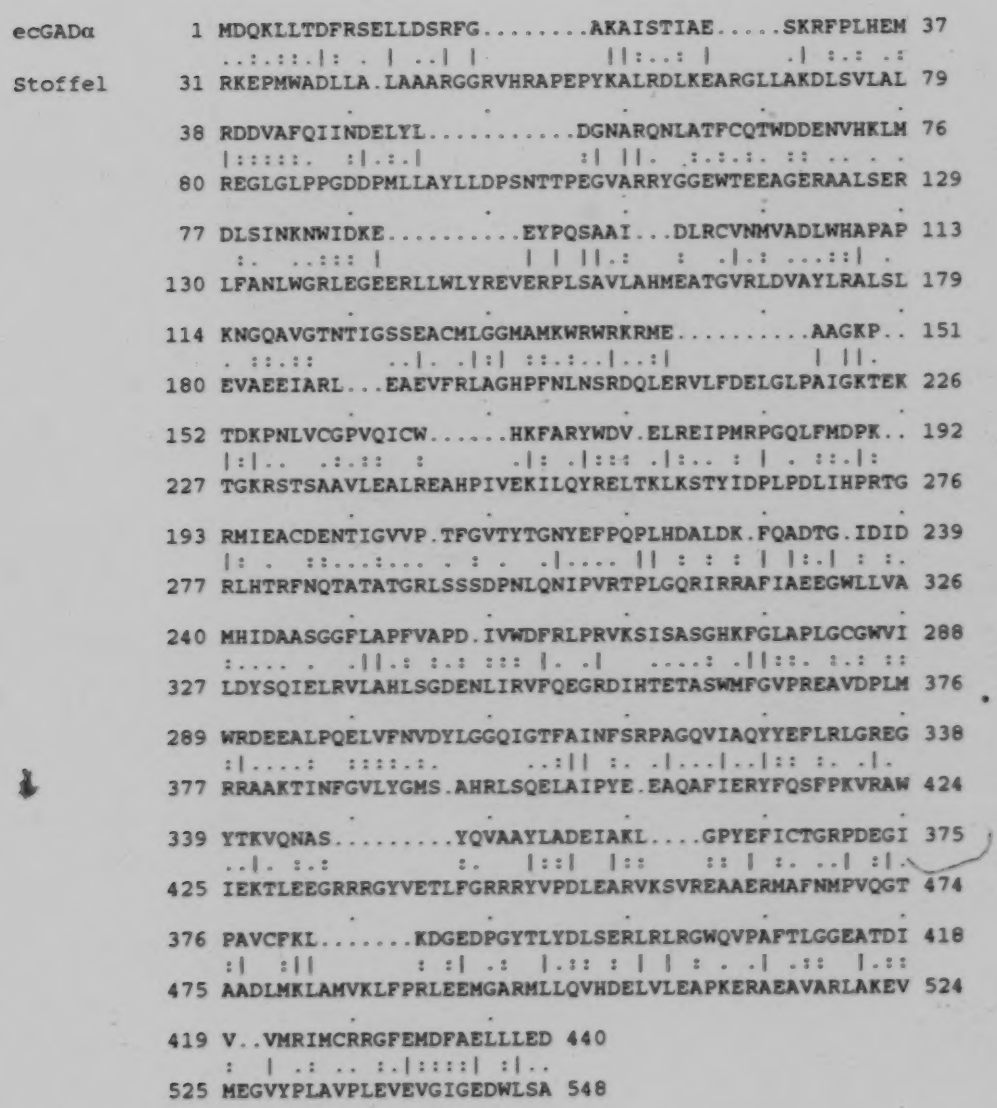

FIG. A-2-4. Best Fit alignment of ecGADα versus Stoffel fragment polypeptides.

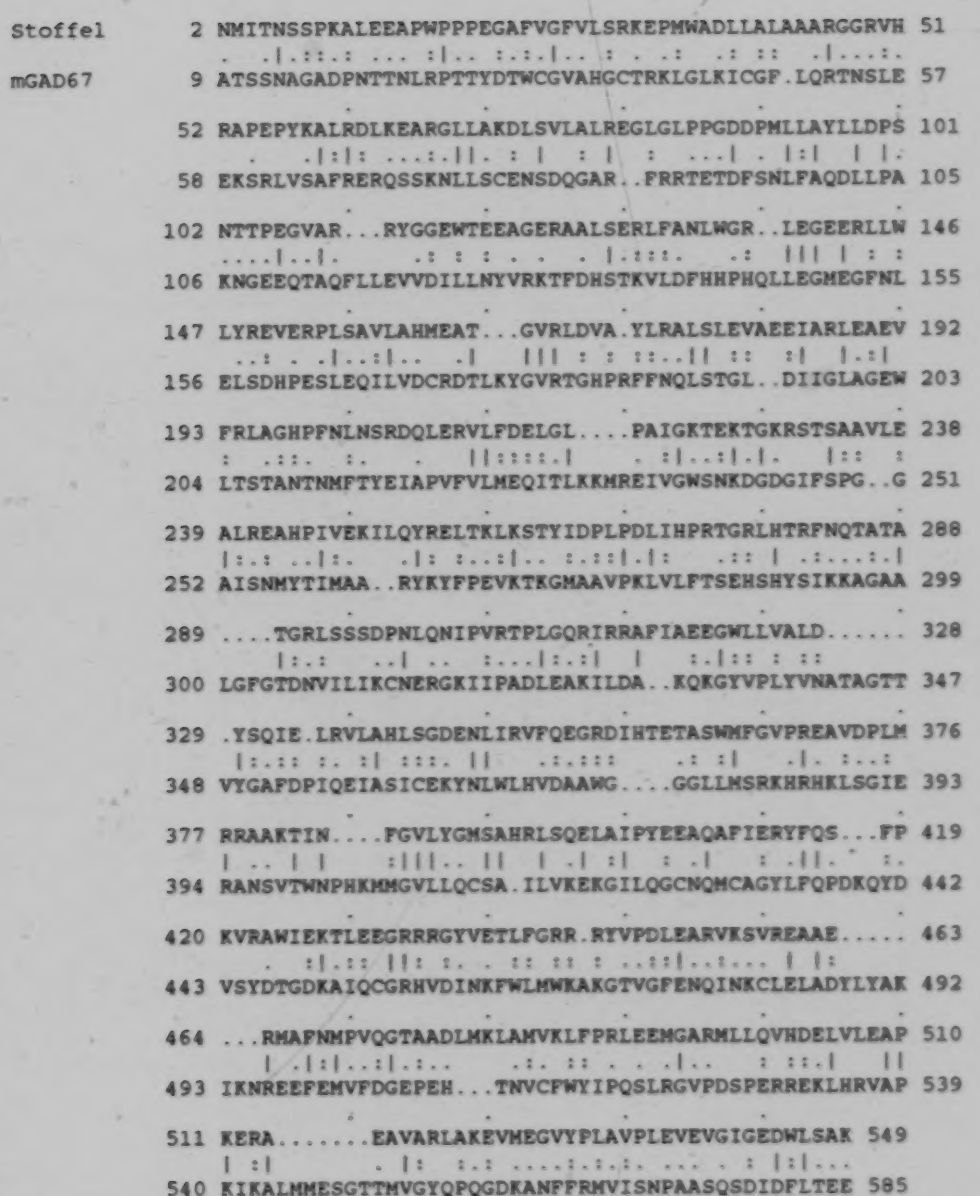

FIG. A-2-5. Best Fit alignment of Stoffel fragment versus mGAD67 polypeptides.

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2. Smith, D.K., Korbutt, G.S., Suarez-Pinzon, W.L., Kao, D., Rajotte, R.V. Elliott, J.F. (1997). IL-4 or IL-10 expressed from adenovirus transduced syngeneic islet grafts fails to prevent ß cell destruction in diabetic NOD mice. *Transplantation*. **64**, 1040-1049.

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